PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 43179/14/AJC	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. **PCT**/NZ2004/000333	International filing date *(day/month/year)* 22 December 2004	Priority date *(day/month/year)* 22 December 2003

International Patent Classification (IPC) or national classification and IPC

Int. Cl.

C12N 9/02 (2006.01) ***C12N 15/52*** (2006.01)

Applicant

AGRESEARCH LIMITED et al

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. [X] *(sent to the applicant and to the International Bureau)* a total of **161** sheets, as follows:
      - [ ] sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      - [ ] sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.

   b. [ ] *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or table related thereto, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).
4. This report contains indications relating to the following items:

[X]	Box No. I	Basis of the report
[ ]	Box No. II	Priority
[X]	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
[X]	Box No. IV	Lack of unity of invention
[X]	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
[ ]	Box No. VI	Certain documents cited
[ ]	Box No. VII	Certain defects in the international application
[ ]	Box No. VIII	Certain observations on the international application

Date of submission of the demand 27 May 2005	Date of completion of this report 24 February 2006
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer **PHILIPPA WYRDEMAN** Telephone No. (02) 6283 2554

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/NZ2004/000333

Box No. I Basis of the report

1. With regard to the **language,** this report is based on:

[X] The international application in the language in which it was filed

[ ] A translation of the international application into , which is the language of a translation furnished for the purposes of:

[ ] international search (under Rules 12.3(a) and 23.1 (b))

[ ] publication of the international application (under Rule 12.4(a))

[ ] international preliminary examination (Rules 55.2(a) and/or 55.3(a))

2. With regard to the **elements** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

[ ] the international application as originally filed/furnished

[X] the description:

pages as originally filed/furnished
pages* **1-90** received by this Authority on **4 October 2005** with the letter of 3 October 2005
pages* received by this Authority on with the letter of

[X] the claims:

pages as originally filed/furnished
pages* as amended (together with any statement) under Article 19
pages* **91-95** received by this Authority on **21 February 2006** with the letter of 21 February 2006
pages* received by this Authority on with the letter of

[X] the drawings:

pages **1/56-56/56** as originally filed/furnished
pages* received by this Authority on with the letter of
pages* received by this Authority on with the letter of

[X] a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing.

3. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages
[ ] the claims, Nos.
[ ] the drawings, sheets/figs
[ ] the sequence listing *(specify)*:
[ ] any table(s) related to the sequence listing *(specify)*:

4. [ ] This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

[ ] the description, pages
[ ] the claims, Nos.
[ ] the drawings, sheets/figs
[ ] the sequence listing *(specify)*:
[ ] any table(s) related to the sequence listing *(specify)*:

* *If item 4 applies, some or all of those sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/NZ2004/000333

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos: **1-13 partially, 15-40 partially, and 14**

because:

☐ the said international application, or the said claims Nos.
relate to the following subject matter which does not require an international preliminary examination *(specify)*:

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos.
are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos.
are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*

☒ no international search report has been established for said claim Nos. **1-13 partially, 15-40 partially, and 14**

☐ A meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ Furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.
- ☐ Furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.
- ☐ Pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13*ter*.1(a) or (b) and 13*ter*.2.

☐ A meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions, and such tables were not available to the International Preliminary Examining Authority in a form and manner acceptable to it

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See Supplemental Box for further details.

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation to restrict or pay additional fees the applicant has, within the applicable time limit:

   ☒ restricted the claims

   ☐ paid additional fees

   ☐ paid additional fees under protest and, where applicable, the protest fee

   ☐ paid additional fees under protest but the applicable protest fee was not paid

   ☐ neither restricted the claims nor paid additional fees

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is:

   ☐ complied with.

   ☒ not complied with for the following reasons:

   The Applicant's comments regarding lack of unity of invention in the attachment accompanying the letter of 3 October 2005 and 21 February 2006 have been considered.

   PCT Rules 13.1 and 13.2 set forth the requirements for establishing unity of invention. In particular rule 13.2 defines what constitutes a 'special technical feature'. In summary unity, is considered to exist when molecules share a common property or activity or structure or structural element, and the common property or activity or structure is a contribution over the prior art.

   With regard to the present application, the fact that the genes are implicated in the lolitrem biosynthesis is not considered to be a special technical feature because a group of genes with this property have been previously disclosed (see Young, C. et al (2003) Molecular Breeding of Forage and Turf, Third International Symposium, May 18-22 2003, Dallas, Texas, USA, Poster#64: 'Molecular cloning and genetic analysis of a fungal endophyte symbiosis expressed gene cluster for lolitrem biosynthesis'). It is not a requirement that the prior art disclosing genes involved in lolitrem biosynthesis be enabling for the isolation of other genes with this property. The fact that genes with this property are known means that this property does not constitute a contribution over the prior art.

   Also the genes do not share a common structure or structural element or any significant sequence homology, therefore there is no unifying structural feature that can be considered to be a special technical feature common to all the genes.

   (Continued in Supplemental Box I)

4. Consequently, this report has been established in respect of the following parts of the international application:

   ☐ all parts.

   ☒ the parts relating to claims Nos. **1-13 and 15-40 as they relate to SEQ ID NOS: 11 and 12**

Box No. V **Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	**1-13, 15-40**	**YES**
	Claims		**NO**
Inventive step (IS)	Claims	**1-13, 15-40**	**YES**
	Claims		**NO**
Industrial applicability (IA)	Claims	**1-13, 15-40**	**YES**
	Claims		**NO**

2. Citations and explanations (Rule 70.7)

The claims are directed to nucleotide/polypeptide sequences (SEQ ID Nos: 11 and 12), obtained from the fungal endophyte *Neotyphodium lolii*, defining a putative P450 monooxygenase that is proposed to be involved in lolitrem biosynthesis. In that the closest prior art is less than 25% identical to the claimed sequences, claims 1-13 and 15-40 are regarded as novel and to involve an inventive step.

The Claims are regarded as industrially applicable under the Articles of the PCT.

Supplemental Box Relating to Sequence Listing

Continuation of Box No. I, item 2:

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this report was established on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [ ] filed together with the international application in electronic form

   [ ] furnished subsequently to this Authority for the purposes of search and/or examination

   [X] received by this Authority as an amendment* on 21 February 2006

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

* *If item 4 in Box No. I applies, the listing and/or table(s) related thereto, which form part of the basis of the report, may be marked "superseded."*

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of: Box IV

Furthermore, the fact that the genes are arranged in a cluster cannot be considered to be unifying, because these clusters simply represent a group of metabolically related genes in a structure that is well known and understood with respect to secondary metabolites in fungi.

Although the only relevant test for unity of invention is as set forth in PCT Rules 13.1 and 13.2, the Applicant has noted that two granted US patent documents, claim gene clusters. While this has no bearing on the tests for unity of invention as required by the PCT, it is noted that neither of these two US patent documents claim the individual genes of the cluster, rather the claims are drawn to the sequence of the cluster *per se*.

Consequently, the present application is directed to multiple inventions as indicated in the previous opinion and reiterated again below.

Continuation of: Box No. IV

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

Note that Rule 13.2 states that where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only where there is a technical relationship among those inventions involving one or more of the same corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The ISA has identified 11 separate inventions:

Invention 1: ltmG (SEQ ID NOs: 1, 2, 17 and 18).

Invention 2: ltmM (SEQ ID NOs: 3, 4, 19, and 20).

Invention 3: ltmK (SEQ ID NOs: 5, 6, 21, and 22).

Invention 4: ltmC (SEQ ID NOs: 7 and 8).

Invention 5: ltmP (SEQ ID NOs: 9 and 10).

Invention 6: ltmJ (SEQ ID NOs: 11 and 12).

Invention 7: ltmQ (SEQ ID NOs: 13 and 14).

Invention 8: ltmD (SEQ ID NOs: 15 and 16).

Invention 9: cluster SEQ ID NO: 23.

Invention 10 cluster SEQ ID NO: 24.

Invention 11 cluster SEQ ID NO: 25.

The claims are directed to nucleotide/polypeptide sequences, obtained from N. lolii and E. festucae, defining enzymes proposed to be involved in lolitrem biosynthesis. Although all of the sequences share the feature that they are proposed to be involved with the lolitrem pathway, this does not represent a special technical feature.

Firstly, this feature cannot be a special technical feature because it is not novel. The lolitrem biosynthesis pathway (and members thereof) is known:

1. Young, C. et al (2003) Molecular Breeding of Forage and Turf, Third International Symposium, May 18-22 2003, Dallas, Texas, USA, Poster#64: "Molecular cloning and genetic analysis of a fungal endophyte symbiosis expressed gene cluster for lolitrem biosynthesis".

(Continued in Supplemental Box II)

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of: Box IV

Secondly, this feature cannot be regarded as a special technical feature as there are no structural features or enzymatic properties that are representative of a single group of structurally or functionally related proteins/nucleic acids. In particular, none of the claimed sequences could be used to obtain the others, and the sequences do not represent a single class of genes nor do they share any significant homology.

With respect to the clusters (SEQ ID NOs: 23, 24, and 25), these clusters simply represent a group of metabolically related genes in a structure that is well known and understood with respect to secondary metabolites in fungi (see the citation above).

Where there is some homology between subgroup members, it is also appropriate to apply the Markush approach to subgroups of the claimed inventions. For example, members of the P450 subgroup claimed (ie SEQ ID NOs: 5, 6, 9, 10, 11, 12, 13, 14, 21, 22) share some homology. However, this homology is also present in other known members of the P450 family. As such, there is no common novel structure present in all of the sequences and there is no single recognised class or group of compounds embracing all the sequences claimed. Thus according to Markush, it is appropriate to classify the sequences in terms of the 11 individual groups and thus these groups represent 11 different inventions.

INDOLE-DITERPENE BIOSYNTHESIS

TECHNICAL FIELD

The present invention relates to the biosynthesis of indole diterpene compounds. In particular, the invention relates to genes encoding enzymes considered responsible for the synthesis of lolitrems.

BACKGROUND ART

Indole-Diterpenes

The indole-diterpenes are a large, structurally diverse group of natural products principally found in filamentous fungi notably of the genera *Penicillium*, *Aspergillus*, *Claviceps*, *Epichloë* and *Neotyphodium* (Steyn and Vleggaar 1985; Mantle 1987; Scott et al. 2003). They may be classified into the following structural sub-groups, such as the simple indole-diterpenes expemplified by emindoles, paxilline, paspaline, and terpendoles (Huang et al., 1995; Tomoda et al., 1995; Gatenby et al., 1999) and the more complex prenyl and diprenyl derivatives of the indole moiety. These complex indole-diterpenes can in turn be classified into further sub-groups such as lolitrems (exemplified by lolitrem B, lolitriol, lolicines, lolilline), penitrems (exemplified by penitrems A-F, pennigritrem, penitremone A-C) (Steyn and Vleggar, 1985), janthitrems (including the very similar shearinines, exemplified by janthitrems A-G, shearinines A-C) (Belofsky et al., 1996), aflatrem, sulpinines (exemplified by sulpinines A & B, Laakso et al., 1992), nodulisporic acid (Ondeyka et al., 1997) and thiersinines (Li et al., 2002), These metabolites all have a common core structure comprised of a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole moiety derived from either tryptophan or a tryptophan precursor (Acklin et al. 1977; de Jesus et al. 1983; Laws and Mantle 1989). Further complexity of the carbon skeleton is achieved by additional prenylations, different

patterns of ring substitutions and different ring stereochemistry. Many of these compounds are potent mammalian tremorgens (Cole and Cox 1981) while others are known to confer anti-insect activity (Gloer 1995).

Paxilline Biosynthesis

Until recently, very little was known about the pathways for the biosynthesis of the indole-diterpenes, although putative biosynthetic schemes have been proposed on the basis of chemical identification of likely intermediates from the organism of interest and related filamentous fungi (Mantle and Weedon 1994; Munday-Finch et al. 1996; Gatenby et al. 1999). The recent cloning and characterization of a cluster of genes from *Penicillium paxilli* required for the biosynthesis of paxilline has provided for the first time an insight into the genetics and biochemistry of indole-diterpene biosynthesis (Young et al. 2001).

Key genes identified in this cluster include a GGPP synthase (*paxG*), a FAD-dependent monooxygenase (*paxM*), a prenyl transferase (*paxC*) and two cytochrome P450 monooxygenases, *paxP* and *paxQ*. Deletion of *paxG* resulted in mutants that were paxilline negative, confirming that this gene is essential for paxilline biosynthesis (Young et al. 2001). Targeted deletion of *paxM* and *paxC* in *P. paxilli* also result in mutants that are defective in paxilline biosynthesis (B. Scott, L. McMillan, J. Astin, C. Young, E. Parker, unpublished results). It is proposed that *PaxM* and *paxC* are required to catalyse the addition of indole-3-glycerol phosphate to GGPP and subsequent cyclisation to form the first stable indole-diterpene, possibly paspaline (Parker and Scott 2004). Deletion of *paxP* and *paxQ* give rise to strains that accumulate paspaline and 13-desoxypaxilline, respectively, suggesting that these are the substrates for the corresponding enzymes (McMillan et al. 2003). Overall, these results establish that at least 5 genes are required for the biosynthesis of paxilline in *P. paxilli*.

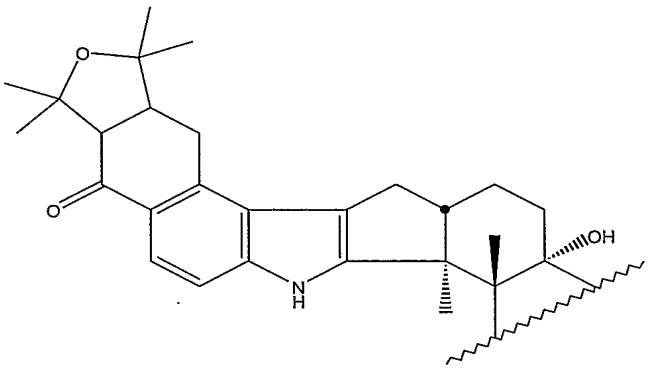
The identification of a geranyl-geranyl diphosphate (GGPP) synthase gene (*paxG*) within this cluster, and confirmation by deletion analysis that it is necessary for paxilline biosynthesis, suggest that the synthesis of GGPP is one of the first steps in the synthesis of this indole-diterpene (Young *et al.* 2001). *P. paxilli*, like *Gibberella fujikuroi* (Mende *et al.* 1997: Tudzynski and Hölter 1998), recently renamed *Fusarium fujikuroi* (O'Donnell *et al.* 1998), has two GGPP synthase genes, but the second, *ggsI*, is unable to complement the *paxG* deletion, presumably because of cellular partitioning of the two enzymes (Young *et al.* 2001). The synthesis of paxilline is predicted to involve several oxygenation steps (Munday-Finch *et al.* 1996), and the presence within the cluster of genes for two FAD-dependent monooxygenases (*paxM* and *paxN*) and for two cytochrome P450 monooxygenases (*paxP* and *paxQ*) is consistent with this chemistry (Young *et al.* 2001).

The only other fungal diterpene gene cluster reported to date is that for the biosynthesis of gibberellins in *Fusarium fujikuroi* (teleomorph *Gibberella fujikuroi*)(Tudzynski and Hölter 1998). This cluster also includes a GGPP synthase gene, *ggs-2*, required for the first committed step in gibberellin biosynthesis. Interestingly, both fungal species contain an additional copy of a GGPP synthase gene, *ggs1* in *P. paxilli* (Young et al. 2001) and *ggs-1* in *F. fujikuroi* (Mende et al. 1997). This suggests that the presence of two copies of GGPP synthases could be a molecular signature for diterpene biosynthesis in filamentous fungi, one copy being required for primary metabolism and the second for secondary (diterpene) metabolism. Given that genes for secondary metabolite biosynthesis in fungi are generally organised in clusters (Keller and Hohn 1997), molecular cloning of GGPP synthases combined with chromosome walking provides a rapid strategy for cloning new indole-diterpene gene clusters.

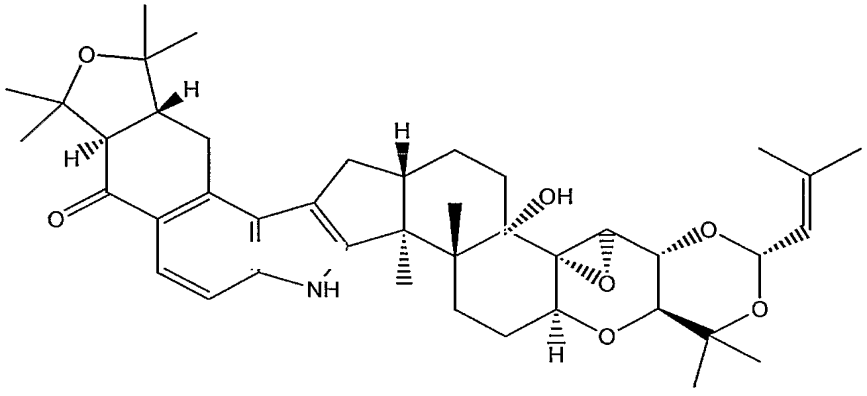
Lolitrems

Epichloë/Neotyphodium endophytes are a group of Clavicipitaceous fungi (Clavicipitaceae, Ascomycota) that form symbiotic associations with temperate climate grasses such as perennial ryegrass and tall fescue (Schardl 2001; Scott 2001). The plant provides nutrients for the endophyte and a means of dissemination through the seed. The endophyte protects the host from biotic (e.g. insect and mammalian herbivory) and abiotic stress (e.g. drought). Fungal synthesis of secondary metabolites appears to be the main mechanism for protection of the symbiotum from herbivory.

The ability of *Epichloë/Neotyphodium* endophytes to synthesize bioprotective metabolites *in planta* constitutes a major ecological benefit for the symbiotum (Schardl 1996). Metabolites identified to date include both anti-insect (e.g. peramine and lolines) and anti-mammalian (ergot alkaloids and indole-diterpenes)(Bush et al. 1997). However, from an agricultural perspective endophyte production of mammalian toxins such as the indole-diterpenes of the lolitrem group with the moiety:



or derivatives thereof, and in particular lolitrem B:



are detrimental to grazing livestock. Consequently, there is considerable commercial interest in developing associations containing endophytes that are not toxic to mammals (Fletcher 1999; Popay et al. 1999).

The lolitrems are produced by the *Epichloë/Neotyphodium* endophytes in association with temperate grass species (Gallagher et al., 1984). These fungi are often found as an infection in perennial ryegrass (*Lolium perenne*) and tall fescue grasses (*Festuca arundinacea*).

Endophytes are symbiotic fungi and are prevalent in New Zealand pastures. The fungal metabolites from these endophytes are thought to serve as chemical defence systems for the fungi that produce them. They may also be of use in protecting the food source from consumption by other organisms (US 4,973,601).

However some of these fungi also pose a problem in that, at least lolitrem B, is known to be the main causative agent in ryegrass staggers (Fletcher and Harvey, 1981). This is a condition in which animals grazing on endophyte infected pastures develop ataxia, tremors, and hypersensitivity to external stimuli. The lolitrem neurotoxin (staggers) reaction is long acting but is however completely reversible (Smith et al 1997, McLeay et al 1999). The time course of tremors induced by lolitrem B is dramatically different from that of other indole diterpenes, for example paxilline and analogues. Paxilline analogues induce tremors of rapid onset and short duration while tremors induced by lolitrem derivatives take hours to reach maximum intensity and last for days.

The mechanism by which lolitrem B and related indole-diterpenes cause

tremorgenicity in mammals is not well defined but biochemical and clinical studies indicate that these effects are due in part to effects on receptors and interference with neurotransmitter release in the central and peripheral nervous system (Selala et al. 1991). Some have been shown to potentiate chloride currents through $GABA_A$ receptor chloride channels heterologously expressed in *Xenopus* oocytes (Yao et al. 1989). Many of the indole-diterpenes are potent inhibitors of high conductance Ca^{2+}-activated K^+ (maxi-K) channels (Knaus et al. 1994; McMillan et al. 2003)

It would therefore be useful if the genes involved in the biosysthesis of indole diterpenes related to lolitrems could be identified as this would provide information useful in: manipulating this biosynthetic pathway; producing indole diterpenes related to lolitrems; identifying mutations in endophytes which produce indole diterpenes related to lolitrems.

All references, including any patents or patent applications cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

It is acknowledged that the term 'comprise' may, under varying jurisdictions, be attributed with either an exclusive or an inclusive meaning. For the purpose of this specification, and unless otherwise noted, the term 'comprise' shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements. This rationale will also be used when the term 'comprised' or 'comprising' is used in relation to one or more steps in a method or process.

It is an object of the present invention to address the foregoing problems or at least to provide the public with a useful choice.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

DISCLOSURE OF INVENTION

According to one aspect of the present invention there is provided an isolated nucleic acid molecule having a nucleic acid sequence selected from the group consisting of:

a) SEQ ID NOs 1, 3, 5, 17, 19, 21, 7, 9, 11, 13, 15, 52 and 54 or a combination of these sequences;

b) SEQ ID NOs 23, 24 and 25;

c) a functional fragment or variant of the sequences in a) or b);

d) a complement to the sequences in a), b) or c).

In some embodiments the isolated nucleic acid molecule may have at least 70% sequence homology to a nucleic acid molecule substantially as described above.

More preferably the isolated nucleic acid molecule may have:

- at least 80% sequence homology or
- at least 90% sequence homology or
- at least 95% sequence homology to a nucleic acid substantially as described above.

Most preferably the isolated nucleic acid molecule may have at least 99% sequence homology to a nucleic acid molecule substantially as described above.

According to another aspect of the present invention there is provided an isolated polypeptide having an amino acid sequence selected from the group consisting of:

a) SEQ ID NOs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 53 and 55 or a combination of these sequences;

b) A functional fragment or variant of the sequences listed in a).

In some embodiments the isolated polypeptide molecule may have at least 70% sequence homology to a polypeptide substantially as described above.

More preferably the isolated nucleic acid molecule may have:

- at least 70% sequence homology to a polypeptide or
- at least 80% sequence homology to a polypeptide or
- at least 90% sequence homology to a polypeptide or
- at least 95% sequence homology to a polypeptide substantially as described above.

Most preferably the isolated polypeptide molecule may have at least 99% sequence homology to a polypeptide substantially as described above.

According to another aspect of the present invention there is provided a primer capable of binding to a nucleic acid molecule substantially as described above.

Most preferably a primer having a nucleotide sequence which comprises at least substantially 15-20 contiguous nucleotides of a nucleic acid molecule selected from the group consisiting of: SEQ ID NOs. 1, 3, 5, 17, 19, 21, 7, 9, 11, 13, 15, 52 and 54.

In some preferred embodiments there may be a primer having a nucleotide sequence selected from the group consisting of SEQ ID NOs 26-51.

According to another aspect of the present invention there is a probe capable of binding to a nucleic acid molecule substantially as described above.

The use of a probe capable of binding to a nucleic acid molecule substantially as described above to identify at least one gene of the lolitrem gene cluster in an endophyte.

The said endophyte may preferably be derived from the *Epichloë* or *Neotyphodium* genus.

An isolated nucleic acid molecule which is able to stringently hybridize to a nucleic acid molecule substantially as described above.

An isolated nucleic acid molecule substantially as described above wherein the molecule is a primer.

An isolated nucleic acid molecule substantially as described above wherein the molecule is a probe.

A method for identifying mutations in the lolitrem gene cluster of an endophyte exhibiting useful phenotypic traits, characterized by the steps of:

a) identifying at least one gene in the lolitrem gene cluster of an endophyte;

b) sequencing the gene(s) identified at a);

c) comparing the sequence at b) to SEQ ID NOs 1, 3, 5, 17, 19, 21, 7, 9, 11, 13, 15, 52 and 54 or a combination of these sequences to ascertain any differences in nucleotide sequence.

Preferably, these phenotypic traits may include non-tremorgenic strains/isolates or strains with increased insecticidal activity including those that produce lolitrem intermediates and/or shearinines and/or janthitrems and/or terpendoles and/or which produce an effect or effects selected from: a less toxic effect, a more toxic effect, a desired agricultural effect, a desired biochemical effect, a desired neurological effect, a desired insecticidal effect, and combinations thereof.

An endophyte in which at least one of the genes in the lolitrem gene cluster has been mutated or otherwise disrupted to manipulate the indole diterpene biosynthetic pathway.

Preferably these include but are not limited to: lolitrem intermediates and/or shearinines, and/or janthitrems, and/or terpendoles.

Preferably, indole diterpenes are lolitrem compounds.

The use of a nucleic acid molecule substantially as described above to produce an indole diterpene, enzyme, intermediate or other chemical compound associated with the indole diterpene biosynthetic pathway.

The use of a nucleic acid molecule substantially as described above to study the indole diterpene pathway.

A construct which includes a nucleic acid molecule substantially as described above.

A host cell which includes a non-endogenous nucleic acid molecule substantially as described above.

An endophyte which includes a non-endogenous nucleic acid molecule substantially as described above.

The use of a polypeptide substantially as described above to catalyze *in vitro* or *in vivo* a reaction involved in the biosynthesis of an idole diterpene.

A kit for identifying the lolitrem gene cluster which includes a probe.

A kit for identifying the lolitrem gene cluster which includes at least one primer pair.

A method of manipulating the indole diterpene biosynthetic pathway characterized by the step of altering a nucleic acid substantially as described above to produce a gene encoding a non-functional polypeptide.

The use of a gene produced by the method substantially as described above to manipulate the indole diterpene biosynthetic pathway.

An expression system which includes a non-endogenous nucleic acid molecule substantially as described above.

The use of an expression system substantially as described above to produce indole diterpene, enzyme, intermediate or other chemical compound associated with the indole diterpene biosynthetic pathway.

The use of a primer substantially as described above to amplify a nucleic acid molecule.

A plant including a cell which includes a non-endogenous nucleic acid molecule substantially as described above.

A plant substantially as described above wherein the plant is a grass.

A plant substantially as described above wherein the plant is a rye grass.

A plant substantially as described above wherein the cell is present as an endophyte.

The use of an isolated nucleic acid molecule in the biosysthesis of an indole diterpene.

Throughout this specification the terms 'pax' and 'ltm' refer to orthologous genes, i.e. genes present in two different species which are different to one another but to a certain extent correspond (having homology) as they were derived from a common ancestor. The prefix used relates to the compound expressed by the gene, i.e. paxiline in the case of pax and lolitrem in the case of *ltm*.

Further, for the purposes of the specification, the terms 'biosynthesis' or 'biosynthetic' refer to the production of a chemical compound in a living organism via processes of that organism.

The alteration of a nucleic acid molecule to produce a gene expressing a non-functional polypeptide may be achieved in a variety of different ways which may include mutagenesis or gene silencing using techniques well known in the art.

The term 'manipulate' or 'manipulating' as used herein refers to the ability to up-regulate or down-regulate or otherwise control the indole diterpene biosynthetic pathway.

The term 'non-endogenous nucleic acid' as used herein refers to a nucleic acid molecule that does not naturally occur within a organism.

The term 'expression system' refers to any cell which can be used to express the polypeptides encoded by at least one nucleic acid molecule of interest. In general suitable cells for use as expression systems include bacteria, yeast, fungi, plants and animal cells.

The term 'indole diterpene' refers to any compound having a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole moiety derived from either tryptophan or a tryptophan precursor. Most preferably the term indole diterpene refers to a lolitrem compound.

The term 'non-functional' refers to a polypeptide which is incapable of acting as an

enzyme in indole diterpene biosynthesis.

It is to be clearly understood that the invention also encompasses peptide analogues, which include but are not limited to the following:

1. Compounds in which one or more amino acids is replaced by its corresponding D-amino acid. The skilled person will be aware that retro-inverso amino acid sequences can be synthesised by standard methods; see for example Choreo and Goodman, 1993;

2. Peptidomimetic compounds, in which the peptide bond is replaced by a structure more resistant to metabolic degradation. See for example Olson et al, 1993; and

3. Compounds in which individual amino acids are replaced by analogous structures for example, gem-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge.

The use of such alternative structures can provide significantly longer half-life in the body, since they are more resistant to breakdown under physiological conditions.

Methods for combinatorial synthesis of peptide analogues and for screening of peptides and peptide analogues are well known in the art (see for example Gallop et al, 1994; Hogan, 1997).

For the purposes of this specification, the term "peptide and peptide analogue" includes compounds made up of units which have an amino and carboxy terminus separated in a 1,2, 1,3, 1,4 or larger substitution pattern. This includes the 20 naturally-occurring or "common" α-amino acids, in either the L or D configuration, the biosynthetically-available or "uncommon" amino acids not usually found in proteins, such as 4-hydroxyproline, 5-hydroxylysine, citrulline and ornithine; synthetically-

derived α-amino acids, such as α-methylalanine, norleucine, norvaline, Cα- and *N*-alkylated amino acids, homocysteine, and homoserine; and many others as known in the art.

It also includes compounds that have an amine and carboxyl functional group separated in a 1,3 or larger substitution pattern, such as β-alanine, γ-amino butyric acid, Freidinger lactam (Freidinger *et al*, 1982), the bicyclic dipeptide (BTD) (Freidinger *et al*, 1982; Nagai and Sato, 1985), amino-methyl benzoic acid (Smythe and von Itzstein, 1994), and others well known in the art. Statine-like isosteres, hydroxyethylene isosteres, reduced amide bond isosteres, thioamide isosteres, urea isosteres, carbamate isosteres, thioether isosteres, vinyl isosteres and other amide bond isosteres known to the art are also useful for the purposes of the invention.

A "common" amino acid is a L-amino acid selected from the group consisting of glycine, leucine, isoleucine, valine, alanine, phenylalanine, tyrosine, tryptophan, aspartate, asparagine, glutamate, glutamine, cysteine, methionine, arginine, lysine, proline, serine, threonine and histidine. These are referred to herein by their conventional three-letter or one-letter abbreviations.

An "uncommon" amino acid includes, but is not restricted to, one selected from the group consisting of D-amino acids, homo-amino acids, N-alkyl amino acids, dehydroamino acids, aromatic amino acids (other than phenylalanine, tyrosine and tryptophan), ortho-, meta- or para-aminobenzoic acid, ornithine, citrulline, norleucine, α-glutamic acid, aminobutyric acid (Abu), and α-α disubstituted amino acids.

The term "nucleic acid molecule" as used herein may be an RNA, cRNA, genomic DNA or cDNA molecule, and may be single- or doublestranded. The nucleic acid molecule may also optionally comprise one or more synthetic, non-natural or altered nucleotide bases, or combinations thereof.

"Primers" are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length, which are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, preferably a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g. by the polymerase chain reaction (PCR) or other nucleic acid amplification methods well known in the art. PCR-primer pairs can be derived from the sequence of a nucleic acid according to the present invention, for example, by using computer programs intended for that purpose such as Primer (Version 0.5© 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Methods for preparing and using probes and primers are described, for example, in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed, vol. 1-3, ed Sambrook et al. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY, 1989.

"Probes" are single-stranded nucleic acid molecules with a known nucleotide sequence which is labelled in some way (for example, radioactively, fluorescently or immunologically), which are used to find and mark a target DNA or RNA sequence by hybridising to it.

The term "vector" as used herein encompasses both cloning and expression vectors. Vectors are often recombinant molecules containing nucleic acid molecules from several sources.

A "cloning vector" refers to a nucleic acid molecule originating or derived from a virus, a plasmid or a cell of a higher organism into which another exogenous (foreign) nucleic acid molecule of interest, of appropriate size can be integrated without loss of the vector's capacity for self-replication. Thus vectors can be used to introduce at

least one foreign nucleic acid molecule of interest (e.g. gene of interest) into host cells, where the gene can be reproduced in large quantities.

An "expression vector" refers to a cloning vector which also contains the necessary regulatory sequences to allow for transcription and translation of the integrated gene of interest, so that the gene product of the gene can be expressed.

The term "gene" as used herein refers to a nucleic acid molecule comprising an ordered series of nucleotides that encodes a gene product (i.e. a specific protein).

The term "protein" or "polypeptide" or "peptide" refers to a chain of L-amino acids linked by amide bonds with or without modifications or additions and with sequence encoded by the nucleic acid molecule of the invention, including fragments, mutations and homologs or analogs having the same biological activity e.g. of a specific enzyme. The protein or polypeptide or peptide of the invention can be isolated from a natural source, produced by the expression of a recombinant nucleic acid molecule, or can be chemically synthesized. The amino acids are referred to herein by their conventional three-letter or one-letter abbreviations.

The term "host cell" refers to a cell which is capable of containing a vector or construct and supports the replication and/or expression of the vector or construct. Suitable hosts cells may be prokaryotic cells such as bacteria, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Preferably, the host cells are bacterial cells.

Understandably, the term "host cell" should also be taken to include a transgenic organism which comprises a host cell.

The term "hybridisation" or grammatical variants thereof means the process of joining two complementary strands of DNA or one each of DNA and RNA to form a double stranded molecule.

The term "construct" as used herein refers to an artificially assembled or isolated nucleic acid molecule which includes the gene of interest. In general a construct may include the gene or genes of interest and appropriate regulatory sequences. It should be appreciated that the inclusion of regulatory sequences in a construct is optional for example, such sequences may not be required in situations where the regulatory sequences of a host cell are to be used. The term construct includes vectors but should not be seen as being limited thereto.

The term "nucleic acid amplification technique" as used herein may generally be considered to refer to polymerase chain reaction or PCR however; it may equally refer to other equivalent techniques for amplifying nucleic acids known to those skilled in the art.

The term 'variant' as used herein refers to a nucleic acid molecule or polypeptide wherein the nucleotide or amino acid sequence exhibits substantially 70, 80, 95, or 99% homology with the nucleotide or amino acid sequence as set forth in the sequence listing – as assessed by GAP or BESTFIT (nucleotides and peptides), or BLASTP (peptides), or BLASTX (nucleotides). It should be appreciated that the variant may result from a modification of the native nucleotide or amino acid sequences, or by modifications including insertion, substitution or deletion of one or more nucleotides or amino acids. Where such a variant is desired, the nucleotide sequence of the native DNA may be altered appropriately for example by systhensis of the DNA *de novo*, or by modification of the native DNA, for example by site-specific or cassette mutagenesis. Preferably, where portions of the cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed using techniques standard in the art. Alternatively, a variant may be naturally occurring. The term variant also encompasses homologus sequences which hybridise under stringent conditions to the sequences of the invention.

The term 'variant' also encompasses "conservative substitutions" wherein the

alteration of the nucleotide or amino acid sequences, as set out in the sequence listing of this specification, results in the substitution of a functionally similar amino acid residue - see Creighton (1984).

The term 'fragment nucleic acid molecule' as used herein refers to a nucleic acid molecule which represents a portion of the nucleic acid molecule of the present invention and is therefore less than full length and comprises at least a minimum sequence capable of hybridising stringently with a nucleic acid molecule of the present invention (or a sequence complementary thereto).

A 'fragment polypeptide' as used herein refers to a fragment of a polypeptide which represents a portion of the polypeptide of the present invention and is therefore less than full length and comprises at least a minimum nucleotide sequence capable of hybridising stringently with a polypeptide of the present invention (or a sequence complementary thereto).

The term "isolated" with respect to a nucleic acid molecule or a polypeptide means substantially separated or purified away from contaminating sequences in the cell or organism in which the nucleic acid or polypeptide naturally occurs and includes nucleic acids purified by standard purification techniques as well as nucleic acids prepared by recombinant technology, including PCR technology, and those chemically synthesised.

The term 'functional' refers to either: a nucleic acid molecule which encodes a polypeptide capable of acting as an enzyme in the indole diterpene biosynthetic pathway; or a polypeptide which is capable of acting as an enzyme in the indole diterpene biosynthetic pathway.

Stringent hybridization conditions is a term of art understood by those of ordinary skill in the art. For any given nucleic acid sequence, stringent hybridization conditions are those conditions of temperature, chaotrophic acids, buffer, and ionic strength which

will permit hybridization of that nucleic acid sequence to its complementary sequence and not to substantially different sequences. The exact conditions which constitute "stringent conditions" depend upon the nature of the nucleic acid sequence, the length of the sequence, and the frequency of occurrence of subsets of that sequence within other non-identical sequences. By varying hybridization conditions from a level of stringency at which non-specific hybridization occurs to a level at which only specific hybridization is observed, one of ordinary skill in the art can, without undue experimentation, determine conditions which will allow a given sequence to hybridize only with complemtary sequences. Suitable ranges of such stringency conditions are described in Krause and Aaronson (1991). Hybridization conditions, depending upon the length and commonality of a sequence, may include temperatures of 20°C-65°C and ionic strengths from 5x to 0.1x SSC. Highly stringent hybridization conditions may include temperatures as low as 40-42°C (when denaturants such as formamide are included) or up to 60-65°C in ionic strengths as low as 0.1x SSC. These ranges, however, are only illustrative and, depending upon the nature of the target sequence, and possible future technological developments, may be more stringent than necessary. Less than stringent conditions are employed to isolate nucleic acid sequences which are substantially similar, allelic or homologous to any given sequence.

The preferred nucleic acid molecules and polypeptides of thie present invention methods, and uses of same may have a number of utilities which can include:

- Manipulating the indole diterpene biosynthetic pathway.
- Producing indole diterpene(s), lolitrem(s), enzyme(s), intermediate(s) or other compound(s), associated with the indole diterpene biosynthetic pathway.
- Identifying mutations of these genes in endophytes which:
    - Do not produce, or produce insufficient levels of lolitrem B, to exhibit

toxic effects such as ryegrass staggers; or

- Provide increased insecticidal activity.

- Providing nucleic acid molecules which can be used in constructs for expression of lolitrems or other indole diterpenes or intermediate compounds involved in the indole diterpene biosynthetic pathway.

It should also be appreciated from the above description that there is provided nucleic acid molecules for the biosynthesis of indole diterpene compounds. It will be appreciated further that through knowledge of these molecules, further molecules can be determined that relate to various aspects of the biosynthesis process. Further, it will be appreciated that the genes have a variety of resulting applications such as screening to determine biosynthesis products and manipulation of the genes to create desireable intermediate and end product indole diterpene compounds.

Sequence listings:

ID No.	Corresponding sequence	Corresponding Figure
1.	ltmG nt (N.lolii)	Figure 4
2.	ltmG pp (N.lolii)	Figure 5
3.	ltmM nt (N.lolii)	Figure 6
4.	ltmM pp (N.lolii)	Figure 7
5.	ltmK nt (N.lolii)	Figure 8
6.	ltmK pp (N.lolii)	Figure 9
7.	ltmC nt (N.lolii)	Figure 29
8.	ltmC pp (N.lolii)	Figure 30
9.	ltmP nt (N.lolii)	Figure 31
10.	ltmP pp (N.lolii)	Figure 32
11.	ltmJ nt (N.lolii)	Figure 41
12.	ltmJ pp (N.lolii)	Figure 42
13.	ltmQ nt (N.lolii)	Figure 33
14.	ltmQ pp (N.lolii)	Figure 34
15.	ltmD nt (N.lolii)	Figure 37
16.	ltmD pp (N.lolii)	Figure 38
17.	ltmG nt (E.festucae)	Figure 11
18.	ltmG pp (E.festucae)	Figure 14
19.	ltmM nt (E.festucae)	Figure 12
20.	ltmM pp (E.festucae)	Figure 15
21.	ltmK nt (E.festucae)	Figure 13
22.	ltmK pp (E.festucae)	Figure 16
23.	Cluster 1	Figure 10

24.	Cluster 2	Figure 28
25.	Cluster 3	Figure 40
26.	Primer ggpps27	Table 2
27.	Primer ggpps28	Table 2
28.	Primer ggpps29	Table 2
29.	Primer CY 4	Table 2
30.	Primer CY 5	Table 2
31.	Primer lol 1	Table 2
32.	Primer lol 2	Table 2
33.	Primer lol 3	Table 2
34.	Primer lol 7	Table 2
35.	Primer lol 14	Table 2
36.	Primer lol 15	Table 2
37.	Primer lol 17	Table 2
38.	Primer lol 18	Table 2
39.	Primer lol 28	Table 2
40.	Primer lol 29	Table 2
41.	Primer lol 32	Table 2
42.	Primer lol 34	Table 2
43.	Primer lol 35	Table 2
44.	Primer lol 43	Table 2
45.	Primer lol 48	Table 2
46.	Primer lol 49	Table 2
47.	Primer lol 63	Table 2
48.	Primer lol 79	Table 2
49.	Primer lol 135	Table 2
50.	Primer lol 147	Table 2
51.	Primer lol 148	Table 2
52.	ltmE nt (N.lolii)	Figure 43
53.	ltmE pp (N.lolii)	Figure 44
54.	ltm25nt (N.lolii)	Figure 35
55.	ltm25pp (N.lolii)	Figure 36

BRIEF DESCRIPTION OF DRAWINGS

Further aspects of the present invention will become apparent from the following description which is given by way of example only and with reference to the accompanying drawings in which:

Figure 1. Structure of lolitrem B

Figure 2. Degenerate PCR and Southern hybridisation of the GGPP synthase gene fragments CY28 and CY29 Degenerate PCR analysis using primers (**A**) ggpps27 and ggpps28, and (**B**) ggpps27 and ggpps29. Lane (1) 1 kb+ ladder, (2) *N. lolii* strain Lp19, (3) *E. festucae* strain Fl1, (4) E. *typhina* strain E8 (5) wild-type *P. paxilli*, (6) *P.*

paxilli strain LM662, (7) blank. Southern hybridisation of the ggs fragments. (**C**) probed with fragment CY29 (*ggs1*). (**D**) probed with fragment CY28 (*ltmG*). Lane (1, 4 and 7) *N. lolii* strain Lp19, (2, 5 and 8) *E. festucae* strain Fl1 (3, 6 and 9) *E. typhina* strain E8. Lanes 1-3 are digested with *Eco*RI, lanes 4-6 are digested with *Hin*dIII and lanes 7-9 are digested with *Sst*I. The size standards are in kb.

Figure 3 . *N. lolii* and *E. festucae* lolitrem gene cluster. Physical map of the (**A**) Lp19 and (**B**) Fl1 lolitrem gene cluster. The CY28 PCR fragment used as a probe to isolate lambda clones, is indicated by a dark grey box on (A). Each gene is shown as a black rectangle with intron marked and an arrow above the genes shows the gene direction. The light grey box is a microsatellite with a core sequence of TAATG. The dark grey boxes on (B) are the fragments used to make the *ltmM* knockout construct. The retrotransposons, Tahi and Rua, are shown as lines with arrow heads as the LTR sequences. Each fragment used as a probe is indicated by an oval placed under the region of the probe. (**C**) The *ltmM* knockout construct, pCY39. (**D**) The PCR screen for a knockout in Fl1. Lanes (1) 1kb+ ladder, (2) CYFl1M-28, (3) CYFl1M-142, (4) CYFl1M-61, (5) CYFl1M-151, (6) Fl1, (7) pCY39, (8) H_2O control. The 7-kb *Xho*I fragment used for preparing the complementation construct is also shown.

Figure 4. The nucleotide sequence of *N. lolii* strain Lp19 *ltmG*.

Figure 5. The polypeptide sequence of *N. lolii* strain Lp19 *LtmG*.

Figure 6. The nucleotide sequence of *N. lolii* strain Lp19 *ltmM*.

Figure 7. The polypeptide sequence of *N. lolii* strain Lp19 *LtmM*.

Figure 8. The nucleotide sequence of *N. lolii* strain Lp19 *ltmK*.

Figure 9. The polypeptide sequence of *N. lolii* strain Lp19 *LtmK*.

Figure 10. The nucleotide sequence of *N. lolii* strain Lp19 *ltmG*, *ltmM* and *ltmK* gene cluster.

Figure 11. The nucleotide sequence of *E. festucae* strain Fl1 *ltmG*.

Figure 12. The nucleotide sequence of *E. festucae* strain Fl1 *ltmM*.

Figure 13. The nucleotide sequence of *E. festucae* strain Fl1 *ltmK*.

Figure 14. The polypeptide sequence of *E. festucae* strain Fl1 *LtmG*

Figure 15. The polypeptide sequence of *E. festucae* strain Fl1 *LtmM*

Figure 16. The polypeptide sequence of *E. festucae* strain Fl1 *LtmK*

Figure 17. HPLC analysis of lolitrem alkaloids in leaf extracts of endophyte infected perennial ryegrass. Pseudostem tissue was harvested two months post-infection and analysed for lolitrems by normal phase HPLC. (A) lolitrem B standard (8.4 µg). (B) wild-type strain Fl1 (plant G1137). (C) ltmM mutant PN2303 (plant G1114). (D) ltmM mutant PN2296 (plant G1126). (E) ltmMG mutant PN2301 (plant G1119). (F) ectopic transformant PN2294 (plant G1130). The y-axis shows fluorescence units in millivolts at A440 nm and the x-axis retention time in min. The peak at retention time of 1.9 min corresponds to the solvent front.

Figure 18. Structure of paspaline.

Figure 19. An EST derived nucleic acid fragment from the suppressive subtractive hybridization library with homology to *Penicillium paxili* paxP

Figure 20. An EST derived nucleic acid fragment from the suppressive subtractive hybridization library with homology to *Penicillium paxili* paxP

Figure 21. An EST derived nucleic acid fragment from the suppressive subtractive hybridization library with homology to *Penicillium paxili* paxP

Figure 22. An EST derived nucleic acid fragment from the suppressive subtractive hybridization library with homology to *Penicillium paxili* paxD

Figure 23. An EST derived nucleic acid fragment from the suppressive subtractive hybridization library with homology to *Penicillium paxili* paxD

Figure 24. An EST derived nucleic acid fragment from the suppressive subtractive hybridization library with homology to *Penicillium paxili* paxD

Figure 25. An EST derived nucleic acid fragment from the an *in* vitro culture library with homology to cytochrome P450 monooxygenases

Figure 26. Schematic diagram of PaxP, showing the placement of the EST sequences. The polypeptide sequence is represented as blocks with the size indicated in amino acid residues underneath. The intron placements are numbered above the polypeptide. The primers used for PCR amplification are positioned above the region used for primer design. The EST sequences that are part of the *ltmP* or the *ltmJ* gene are shown as lines below the EST positions. The EST identification numbers (Table 6) have been reduced to the last three numbers.

Figure 27. A physical and genetic map of the Lp19 ltm cluster 2 locus. The five *ltm* genes are shown as arrows, the exons of the *ltm* genes are indicated by boxes under the gene. Selected lambda clones isolated with the *ltmC* and *ltmP* probes are indicated by lines. The fragments used as probes to isolate the lambda clones are shown as boxes above the restriction enzyme map. The fragments initially isolated by IPCR are indicated by shaded boxes.

Figure 28. The nucleotide sequence of *N. lolii* strain Lp19, cluster 2, *ltmP-rev, ltmQ, ltmD, ltmC-rev, ltm25.*

Figure 29. The nucleotide sequence of *N. lolii* strain Lp19 *ltmC.*

Figure 30. The polypeptide sequence of *N. lolii* strain Lp19 *ltmC.*

Figure 31. The nucleotide sequence of *N. lolii* strain Lp19 *ltmP.*

Figure 32. The polypeptide sequence of *N. lolii* strain Lp19 *ltmP.*

Figure 33. The nucleotide sequence of *N. lolii* strain Lp19 *ltmQ.*

Figure 34. The polypeptide sequence of *N. lolii* strain Lp19 *ltmQ.*

Figure 35. The nucleotide sequence of *N. lolii* strain Lp19 *ltm25.*

Figure 36. The polypeptide sequence of *N. lolii* strain Lp19 *ltm25*

Figure 37. The nucleotide sequence of *N. lolii* strain Lp19 *ltmD.*

Figure 38. The polypeptide sequence of *N. lolii* strain Lp19 *ltmD.*

Figure 39. A physical and genetic map of the Lp19 *ltm* cluster 3 locus. The two *ltm* genes, *ltmE* and *ltmJ*, are indicated by arrows, the exons of the *ltm* genes are indicated by boxes under the gene. The lambda clone, λCY324, is shown as an arrow. The primers, lol205 and lol206, used for amplification of the probe fragment are above the gene. The fragment used as a probe to isolate the lambda clones is shown as a box above the restriction enzyme map. The hybridisation with fragments *ltmE* and a fragment spanning *ltmE-ltmJ* was used to extend the map towards the left by IPCR using the restriction enzymes *Cla*I, *Xba*I and *Hin*dIII.

Figure 40. The nucleotide sequence of *N. lolii* strain Lp19, *ltm* cluster 3, *ltmE* and *ltmJ.*

Figure 41. The nucleotide sequence of *N. lolii* strain Lp19 *ltmJ.*

Figure 42. The polypeptide sequence of *N. lolii* strain Lp19 *ltmJ.*

Figure 43. The nucleotide sequence of *N. lolii* strain Lp19 *ltmE.*

Figure 44. The polypeptide sequence of *N. lolii* strain Lp19 *ltmE.*

Figure 45. Making the constructs for complementation of the *paxC* deletion mutant. (A) The pPN1851 construct. (B) The pCY34 construct. The *ltmC* gene from Lp19 was amplified with primers lol235 and lol236, digested with *Ncd* and *Eco*RI and subsequently cloned into pPN1851. The *paxM* promoters are highlighted by a box. (C) The 3.5 kb *Hin*dIII fragment from Lp19 containing *ltmC* was cloned into pUC118 resulting in pCY66. The pCY66 plasmid was used with p1199 in a co-transformation of ABC83 protoplasts. (D) The 2.5 kb *Bc*II fragment from *P. paxilli* contiaing *paxC* was cloned into p1199 resulting in pJA8.

Figure 46. TLC analysis of *paxc* complementation transformants. Indole-diterpenes were extracted from mycelium grown for 7 days in CDYE + TE at 28°C.

All plasmids were used to transform the *paxC* deletion mutant, ABC83. The plasmids were as follows; pll99; pCY66 contained *ltmC* under the control of its native promoter; pJA8 contained *paxC* under the control of its native promoter; pCY34 contained Lp19 *ltmC* gene under the control of the *paxM* promoter. The + under the TLC plate indicates the presence of a band identical in Rf to the paxilline standard, while the + indicates possible paxilline production. 13dP=the mobility of paspaline and 13-desoxypaxilline.

Figure 47. Autoradiographs of Southern analysis of (A) *Eco*RI digested; (B) *Sal*I digested, *N. lolii* strains Lp19, LP5, AR1 and LP14, *E. festucae* strains FL1 and E189, *Neotyphodium* species LpTG2 strain Lp1and *E. typhina* strain E8 hybridised with ^{32}P-labelled *ltmP* amplified with primers lol196 and lol198; *ltmJ* amplified with primers lol205 and lol206, and *ltmE* amplified with primers lol356 and lol341. The sizes of the hybridising bands are shown in kb. (C) A schematic map of the *ltm* cluster 2 and 3 region showing the approximate deletions in Lp14, Lp1 and AR1 as determined by Southern Analysis.

BEST MODES FOR CARRYING OUT THE INVENTION

Example 1. Isolation of nucleic acid fragments containing homology to GGPP synthases from *N. lolii* and *E. festucae*

Fungal strains, *E. coli* strains, plasmids and lambda clones used in this experiment are described in Table 1.

Table 1: Strains, plasmids, and lambda clones.

Strain	PN number	Relevant characteristics	Reference
Lp19	PN2191	*Neotyphodium lolii*	
FI1		*Epichloë festucae*	
E8		*Epichloë typhina*	
CYFI1-M28	PN2303	*E. festucae ΔltmM::hph*	This study
CYFI1-M61	PN2301	E. festucae *ΔltmMG::hph*	This study
CYFI1-M142	PN2296	*E. festucae ΔltmM::hph*	This study
CYFI1-M151	PN2294	*E. festucae ΔltmM::hph* ectopic integration	This study
pCB1004		Amp^R/Hyg^R	Carroll et al 1994
pCY28		209 bp *ltmG* fragment in pGEM-T, Amp^R	This study
pCY29		272 bp *ggsA* fragment in pGEM-T, Amp^R	This study
pCY39		Amp^R / Hyg^R, *ltmM* knockout construct	This study
pGEM-T		Amp^R	Promega
pGEM-T-easy		Amp^R	Promega
pPN1688	PN1688	Amp^R /Hyg^R	This study
pUC118		Amp^R	This study
λCY218		Lp19λGEM12 containing *ltmG*	This study
λCY255		Lp19λGEM12 containing *ltmK*	This study
λCY275		Lp19λGEM12 overlapping λCY255	This study
λCY100		Lp19λGEM12 containing ggsA	This study
G1114		Nui ryegrass, CYFI1-M28	This study
G1119		Nui ryegrass, CYFI1-M61	This study
G1126		Nui ryegrass, CYFI1-M142	This study
G1130		Nui ryegrass, CYFI1-M151	This study
G1137		Nui ryegrass, FI1	This study
G1138		Nui ryegrass, endophyte free	This study

All bacteria were grown in LB medium overnight at 37°C. For maintenance, the fungal cultures were grown on 2.4% potato dextrose (PD; Difco) agar plates at 22°C until suitable growth was attained. For DNA isolation, the fungal strains were grown

in PD broth at 22°C for 5-12 days. The protein sequences of the available fungal GGPPS genes from:

Neurospora crassa al-3, (accession number AAC13867)(Barbato et al. 1996)

S. cerevisiae Bts1 (accession number AAA83662)

P. paxilli paxG (accession number AF279808) (Young et al. 2001), and

Gibberella fujikuroi ggs-1 (accession number CAA65644) (Mende et al. 1997) and

ggs-2 (accession number CAA75568) (Tudzynski and Hölter 1998)

were aligned (Higgins et al. 1994) to determine conserved domains that would be suitable for degenerate primer design. Primers, ggpps27, ggpps28 and ggpps29, were designed to three highly conserved regions taking in to consideration the placement of any known introns. The sequences of these and other primers are shown in Table 2.

Table 2: Primer list

Name	sequence 5' → 3'	amplifies
CY 4	GCT TGG ATC CGA TAT TGA AGG AGC	hph/BamHI
CY 5	TTG GAT CCG GTT CCC GGT CGG CAT	hph/BamHI
ggpps 27	CAY MGI GGT CAR GGT ATG GA	dPCR
ggpps 28	TTC ATR TAG TCG TCI CKT ATY TG	dPCR
ggpps 29	AAC TTT CCY TCI GTS ARG TCY TC	dPCR
lol 1	TGG ATC ATT CGC AGA TAC	*ltmG*
lol 2	GTG TGA GAT TAA GAC GTC	LHS
lol 3	ACC GAC GCC ATT AAT GAG	*ltmG*
lol 7	ACT GGG CAT CTT CCA TAG	*ltmM*/mid
lol 14	ATT AGA GGC ACC GAA CGC	RT-PCR *ltmM*
lol 15	ATC AAG CTG GCT ATC CTC	*ltmP*
lol 17	AAA TAA TGG GCA AGG AGC	KO PstI
lol 18	TGG GAAT TTT GGA AAT GGC	KO PstI
lol 28	GCT CCT TGC CCA TTA TTT	RT-PCR *ltmM*
lol 29	GTC TTG ATC GTC TGC ATC	RT-PCR *ltmP*
lol 32	TGT CCG TGC ATC CAT TGT	*ltmP*
lol 34	CAT AGA GCT AGC TAG AGT	LHS
lol 35	GTT CGG TGC CTC TAA TAC	*ltmM*/mid
lol 43	GAG GAT AGC CAG CTT GAT	RT-PCR *ltmP*
lol 48	GAT TGG TAC CTT GAA GTC GCT AGT	KO KpnI
lol 49	GTA GGG TAC CTC TAG TAC TGC CTC T	KO KpnI

lol 63	TAG CGA ATC ATT GCG TCG	RT-PCR *ltmP*
lol 79	ATG GCT GCC AAT GAC TTT CC	RT-PCR *ltmG*
lol 135	AGG CCA TTT TCG ACA GTT GT	KO integration
lol 147	CCA GCA AGC ATG CAC ATT AC	RHS
lol 148	TGC GTG AGA GAT AAA GCA AG	KO integration
pUC forward	GCC AGG GTT TTC CCA GTC ACG A	
pUChph 3	CTG CAT CAT CGA AAT TGC	hph
pUChph 4	AAA CCG AAC TGC CCG CTG TTC	hph
PUC reverse	GAG CGG ATA ACA ATT TCA CAC AGG	
T7	TAA TAC GAC TCA CTA TAG GG	

Using degenerate primers designed to fungal GGPP synthase genes, a fragment of the expected size (Figure 2A) was amplified from lolitrem producing strains, *Neotyphodium lolii* Lp19, and *Epichloë festucae* Fl1, and from the lolitrem non-producing strain *E. typhina* E8. *P. paxilli* genomic DNA was used as a positive control where two fragments of 330 and 270 bp were amplified, corresponding to paxG, with an intron, and ggs1, without an intron (Figure 2B). Degenerate PCR amplification was performed using primer pairs ggpps27/ggpps28 and ggpps27/ggpps29 with 5 ng of genomic DNA and 4.8 µM of each primer. The amplification conditions were 95°C for 2 min followed by 30 cycles of 95°C for 30 sec, 45°C for 30 sec and 72°C for 1 min, then 1 cycle of 72°C for 5 min. The annealing temperature was also increased to 47°C with a similar amplification result. The resulting products were cloned into pGEM-T easy (Promega). Plasmid DNA was isolated using a BioRad plasmid mini preparation kit. PCR products were purified using a Qiagen PCR purification kit. Fragments were extracted from agarose using the Qiagen gel extraction kit.

The cloned fragments were distinguished using RFLP analysis by amplifying with primers ggpps27 and ggpps28 using standard PCR conditions. The resulting fragments were digested with an appropriate enzyme (*Not*I and *Sau*3AI) and resolved on a 2% agarose gel.

The Lp19 PCR product amplified with primer set ggpps27 and ggpps29 was cloned into pGEM-T easy and sequenced. DNA fragments were sequenced by the

dideoxynucleotide chain termination method (Sanger et al. 1977) using Big-Dye (Version 3) chemistry with oligonucleotide primers (Sigma Genosys) to pGEM-T easy, *N. lolii* and *E. festucae* sequences. Products were separated on an ABI Prism 377 sequencer (Perkin-Elmer).

Sequence data was assembled into contigs using SEQUENCHER version 4.1 (Gene Codes) and analyzed using the Wisconsin Package version 9.1 (Genetics Computer Group, Madison, Wisconsin). Sequence comparisons were performed through Internet Explorer version 6.0 at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/) using the Brookhaven (PDB), SWISSPROT and GenBank (CDS translation), PIR and PRF databases employing algorithms for both local (BLASTX and BLASTP) and global (FASTA) alignments (Pearson and Lipman 1988; Altschul et al. 1990; Altschul et al. 1997).

A BLASTX of the CY29 sequence, showed high sequence similarity (E value of 7e-41) to the *N. crassa* GGPPS (accession number AAC13867) and other GGPPS sequences.

An RFLP screen of the remaining clones revealed a second unique fragment, CY28, that also shows strong similarity to GGPPS genes (the top score was to *P. paxilli Ppggs1* accession number AF279807, Young et al 2001).

CY28 was amplified with ggpps27 and ggpps28 and is therefore a shorter product than the CY29 fragment. The two sequences, CY28 and CY29, share 61.7% identity to each other at the DNA level.

To determine which clone is the GGPP synthase involved in lolitrem biosynthesis, each fragment was hybridized to genomic DNA from the two lolitrem producing strains, Lp19 and Fl1, and the non-producing E8 strain. DNA was transferred to positively charged nylon membrane (Roche) using standard techniques (Sambrook et

al. 1989). Fragments required for radioactive probes were amplified using primer pairs stated in Table 3 below.

Each probe fragment was purified using a Qiagen PCR purification kit and 30ng of DNA was [α-^{32}P]-dCTP radiolabelled using HighPrime (Roche). The labeled probes were spun through a Pharmacia ProbeQuant column before hybridisation. Hybridisations were performed overnight at 65°C and the filters were washed in 2 x SSC, 0.1% SDS at 50°C.

Table 3 Primer combinations for hybridisation probes and RT-PCR analysis

Gene	primer 1 (5')	primer 2 (3')	Size bp genomic (cDNA)	introns amplified
CY28	g27	g28	209	
CY29	g27	g29	272	
ltmG	lol3	lol1	407 (353)	2
ltmM	lol7	lol35	448 (382)	1
ltmK	lol33	lol37	3277	
ltmK	lol15	lol32	416 (365)	5
ltmG	lol79	lol1	630 (525)	1, 2
ltmM	lol7	lol35	448 (382)	1
ltmM	lol14	lol28	576 (414)	2, 3
ltmK	lol29	lol15	1122 (816)	1, 2, 3, 4, 5
ltmK	lol43	lol63	839 (684)	6, 7

The hybridising patterns (Figure2 C & D) showed that CY29 hybridized to all three strains while CY28 hybridised just to the two lolitrem producers, Lp19 and Fl1. This data indicates that CY29 is the orthologue of *P. paxilli* paxG and CY28 the orthologue of *P. paxilli* ggs1.

For reference these genes are named *NlggsA* and *NlltmG* respectively (*ltm* = lolitrem biosynthesis).

Example 2 Isolation of genomic fragments corresponding to *ltm* genes

Using degenerate primers designed to fungal GGPP synthase genes, a fragment of the expected size (Figure2A) was amplified from lolitrem producing strains, *Neotyphodium lolii* Lp19, and *Epichloë festucae* Fl1, and from the lolitrem non-

producing strain *E. typhina* E8 lolitrem non-producing strain. *P. paxilli* genomic DNA was used as a positive control where two fragments of 330 and 270 bp were amplified, corresponding to *paxG*, with an intron, and *ggs1*, without an intron (Figure 2B).

The Lp19 PCR product amplified with primer set ggpps27 and ggpps29 was cloned into pGEM-T easy and sequenced. A BlastX analysis of the CY29 sequence, showed high sequence similarity (E value of 7e-41) to the *N. crassa* GGPPS (accession number AAC13867) and other GGPPS sequences (Table 4). An RFLP screen of the remaining clones revealed a second unique fragment, CY28, that also showed strong similarity to GGPPS genes (the top score was to *P. paxilli Ppggs1* accession number AF279807, Young et al. 2001). CY28 was amplified with ggpps27 and ggpps28 and is therefore a shorter product than the CY29 fragment. The two sequences, CY28 and CY29, share 61.7% identity to each other at the DNA level.

To determine which clone is the GGPP synthase involved in lolitrem biosynthesis, each fragment was hybridized to genomic DNA from the two lolitrem producing strains, Lp19 and Fl1, and the non-producing E8 strain. The hybridising patterns (Figure 2 C & D) showed that CY29 hybridized to all three strains while CY28 hybridised just to the two lolitrem producers, Lp19 and Fl1. This data indicates that CY29 is the orthologue of *P. paxilli ggs1* and CY28 the orthologue of *P. paxilli paxG*.

For reference these genes are named *ggsA* and *ltmG* respectively (*ltm* = lolitrem biosynthesis).

The *ltmG* fragment, CY28, was used as a probe to isolate sequences from a Lp19 λGEM12 genomic library. This region of the genome is under represented in the library with only five clones isolated from ~80,000 plated. A 15.6-kb lambda clone, λCY218 (Figure 3), was completely sequenced and shown to contain a complete copy of the *ltmG* gene.

To obtain further sequence to the left of *ltmG*, the Lp19 λGEM12 library was screened with a probe amplified with primers lol3 and lol1. Hybridization of the library identified one clone λCY219 that contains extra flanking sequence (Figure 3), however, this clone was severely rearranged and only 1051 bp reflects the correct genomic arrangement. Sequence analysis of *ltmG* predicts the presence of two introns (Figure 3). These two introns were confirmed by cDNA analysis with RNA isolated from endophyte infected ryegrass. These introns are conserved in position with two of the four introns found in the *ggs-2* gene from *G. fujikouri* (Tudzynski and Hölter 1998) and two of the three introns found in *P. paxilli paxG* (Young et al. 2001).

The nucleotide sequence of *ltmG* from *N. lolii* strain Lp19 is shown in Figure 4. *LtmG* is predicted to encode a polypeptide of 334 amino acids with an unmodified molecular weight of 37.9 kDa (Table 4). The amino acid sequence of the deduced *N. lolii* LtmG polypeptide is shown in Figure 5. FastA analysis shows that *LtmG* shares 54.1% and 52.6% identity to *N. lolii* GgsA and *P. paxilli* PaxG polypeptide sequences, respectively. *LtmG* contains the five conserved domains found in all prenyl diphosphate synthases (Chen et al. 1994), including the highly conserved aspartate-rich motifs, DDXXD and DDXXN/D, of domains II and V that are proposed binding sites for the isopentenyl diphosphate (IPP) and the allyl isoprenoid substrates. This analysis suggests that *LtmG* is a GGPP synthase required for the first commited step in lolitrem biosynthesis.

Table 4 Analysis of genes in the lolitrem B biosynthesis cluster

Gene	Putative activity	Size (aa)	Transcript size	Intron number	Homologous *pax* gene	Protein identity
ggsA	Geranylgeranyl diphosphate synthase			0	*ggs1*	
ltmG	Geranylgeranyl diphosphate synthase	334	1002+	2	*paxG*	52.6%
ltmM	FAD dependent monooxygenase	472	1416+	3	*paxM*	41.0%
ltmK	cytochrome P450 monooxygenase	533	1599+	7	*paxP*	31.3%

Example 3. Identification of a gene cluster for lolitrem biosynthesis

Adjacent to *ltmG* are two genes, *ltmM* and *ltmK*, (Figure 3) proposed to be a FAD-dependent monooxygenase and cytochrome P450 monooxygenase, respectively.

Sequence analysis and characterisation by cDNA analysis of the *ltmM* gene confirms the presence of three introns (Figure 3).

The first two of these introns are conserved with those found in the *P. paxilli paxM* gene. The third intron is 106 bases, being the largest of the *ltm* introns confirmed. The gene *ltmM* is predicted to encode a polypeptide of 472 amino acids with an unmodified molecular weight of 52.5 kDa (Table 4). The nucleotide sequence of *N. lolii ltmM* and the deduced amino acid sequence of the *LtmM* polypeptide are shown in Figures 6 and 7, respectively. BLASTP analysis showed that *LtmM* shares 41.0% identity to PaxM from *P. paxilli* (E value 5e-94). Clustal W alignment (Higgins et al. 1994) of *LtmM* with PaxM and other closely related polypeptide sequences, identifies the presence of four highly conserved motifs, the dinucleotide binding domain (Wierenga et al. 1986) the ATG motif (Vallon 2000), a GD motif (Eggink et al. 1990) and a G-helix. These motifs are good indicators of a modified Rossman fold, used by many flavoproteins to bind FAD. This analysis suggests that *LtmM*, like PaxM, is a FAD-dependent monooxygenase, possibly an epoxidase, required for epoxidation of GGPP before cyclisation.

Sequence analysis and characterisation by cDNA analysis of *ltmK* identified seven introns, four of which are conserved with *P. paxilli paxP* and three are conserved with *P. paxilli paxQ*. The nucleotide sequence of *N. lolii ltmK* and the deduced amino acid sequence of the LtmK polypeptide are shown in Figures 8 and 9, respectively.

The gene *ltmK* is predicted to encode a polypeptide of 533 amino acids with an unmodified molecular weight of 60.9 kDa (Table 4). LtmK contains the classical signature motifs of cytochrome P450 enzymes, including a haem-binding domain

(Graham-Lorence and Peterson 1996). However, it does not appear to be an orthologue of either PaxP (E value of 9e-62) or PaxQ (E value of 2e-22), two cytochrome P450 enzymes required for paxilline biosynthesis in *P. paxilli* (McMillan et al. 2003), as two other cytochrome P450 genes identified from EST sequences have greater similarity to these genes (see below).

Therefore, *ltmG* forms a gene cluster with an orthologue of *paxM* (*ltmM*) and a cytochrome P450, *ltmK*, of as yet unknown function in lolitrem biosynthesis. The complete nucleotide sequence of this region is shown in Figure10. The corresponding region was sequenced from the *E. festucae* strain FI1 and shown to be 99.9% identical to Lp19, at the DNA level, from the start of *ltmG* to the stop codon of *ltmK*. The nucleotide sequence of *E. festucae ltmG*, *ltmM* and *ltmK* and the deduced amino acid sequence of the corresponding polypeptides LtmG, LtmM and LtmK are shown in Figures 11 to 16, respectively. Comparison of the *E. festucae ltmM* sequence to *N. lolii ltmM* shows two base transitions of A→G at base 91 and T→C at base 249 (position 1 being the A of the ATGstart codon). Only the first transition results in a residue change with a conservative replacement of methionine (in *N. lolii ltmM*) to valine (in *E. festucae ltmM*). The promoter region of *N. lolii ltmM* and *E. festucae ltmM* have two differences, the first, T→C at base -356 is at a *Hin*dIII site that is absent from *E. festucae ltmM* and the second is at base -1038 where a GAGA in Lp19 has expanded to GAGAGA in FI1. *N. lolii ltmK* and *E. festucae ltmK* are identical in sequence.

The DNA sequence flanking the right-hand end of the *ltm* gene cluster contains a high AT content (71.2 %) compared to that of the *ltm* genes at 59.3% AT and, *ggsA* at 40.9% AT. Blast searches of this flanking region reveal sequence similarity to retrotransposons, however, these sequences are very degenerate and no open reading frames are visible.

Example 4 Confirmation that ltmM is essential for lolitrem B function by - Deletion of ltmM and complementation of ltmM mutant

A gene knockout of *ltmM* in the *E. festucae* strain FI1 was used to confirm that *ltmM* is essential for lolitrem production. A replacement construct, pCY39, was used in a gene disruption to recombine into the wild-type genome (Figure3). An initial PCR screen of 159 hygromycin resistant transformants with primers lol148 and lol135, that amplify both the wild-type *ltmM* gene (1.6 kb) and the integrating plasmid (1.4 kb) identified replacements of *ltmM*. Transformants that contain only the integrating plasmid were 'knockout' candidates and were screened further. The second PCR screen was with primer sets to the upstream (lol2 and lol34: 574 bp), *ltmM* gene (lol7 and lol35: 448 bp), or downstream (lol147 and lol15: 317 bp) regions, where absence of the *ltmM* gene confirmed the deletion event. Southern analysis was used to distinguish the true knockouts, of which 3.9% (5/159) contained a single integration of the plasmid. During the screen for a homologous recombination event, a transformant, CYFI1-M61, was identified that has a deletion of *ltmM* and is also deleted beyond *ltmG*, but the extent of the deletion remains uncharacterised.

Two independent knockout strains, CYFI1-M28 (PN2303) and CYFI1-M142 (PN2296), the deletion mutant CYFI1-M61 (PN2301), an ectopic mutant CYFI1-M151 (PN2294), and wild-type FI1 were used to infect endophyte-free perennial ryegrass plants. Each plant was screened for systemic endophyte infection by aniline blue staining confirming normal endophyte associations with the grass. The rate of infection (Table 5) was determined once the plants had established reasonable growth and shows that each strain has a similar infection rate. The endophyte infected plants were grown in a containment green house and were screened for alkaloid production in mid-summer. The alkaloid levels (summarised in Table 5) show that the endophyte strains with a deleted *ltmM* gene (CYFI1-M28, CYFI1-M61 and CYFI1-M142) are unable to produce lolitrem B (Figure17) but the level of ergovaline and peramine

production is consistent with wild-type and the ectopic integrant CYFI1-M151. The knockouts (CYFI1-M28 and CYFI1-M142) and deletion mutant (CYFI1-M61) are also devoid of two smaller peaks, that are assumed to be lolitrem A and E, respectively (Figure17).

A complementation construct for *ltmM*, pCYltmM, was made by cloning a 7 -kb *Xho*I fragment containing 2.2 kb of 5' and 3kb of 3' *ltmM* sequences into pII99. Four random integrants of PN2303 containing this construct were infected into plants and shown to synthesize lolitrems.

Plant Inoculation

Two independent knockout strains, CYFI1-M28 and CYFI1-M142, the deletion mutant CYFI1-M61, an ectopic mutant CYFI1-M151, and wild-type FI1 were used to artificially infect endophyte-free perennial ryegrass plants. Ryegrass cultivar Nui was infected with fungal endophyte according to the procedure of (Latch and Christensen 1985). Four - five weeks after inoculation the plants were checked for systemic endophyte infection by immunoblotting with endophyte antisera and staining pseudostem leaf peels with aniline blue to detect the presence of the endophyte. Plants that were endophyte positive were repotted and allowed to grow under greenhouse conditions. The rate of infection (Table 5) was determined once the plants had established reasonable growth and shows that each strain has a similar infection rate.

Alkaloid Analysis

The endophyte infected plants were grown in a containment green house and were screened for alkaloid production mid-summer. Endophyte infected plant pseudostem material was freeze dried and milled. For lolitrem analysis weighed portions (c. 50 mg) were extracted for 1 hour at ambient temperature with 1 ml of dichloroethane-methanol, 9:1 by volume, in 2 ml polypropylene screw cap vials turning end for end for agitation. The extract was separated by centrifugation and 8 µl portions were

examined for lolitrems by normal phase high performance liquid chromatography (Shimadzu LC-10A system) on Alltima silica 5µ 150 x 4.6 mm columns (Alltech Associates, Deerfield, Il). The mobile solvent was dichloromethane-acetonitrile-water, 860:140:1 by volume, with a flow rate of 1 ml/min. Lolitrems were detected by fluorescence (Shimadzu RF-10A, excitation 265 nm, emission 440 nm). Lolitrem B eluted at approximately 4.5 minutes followed by smaller amounts of other lolitrems. The amount of lolitrem B was estimated by comparison of integrated peak areas with external standards of authentic lolitrem B. The detection limit was estimated as < 0.1 ppm of lolitrem B.

Ergovaline and peramine were analysed by the method of Spiering et al. (2002).

The alkaloid levels (summarised in Table 5) show that the endophyte strains with a deleted *ltmM* gene (CYFI1-M28, CYFI1-M61 and CYFI1-M142) are unable to produce lolitrem B (Table 5) but the level of ergovaline and peramine production is consistent with wild-type and the ectopic integrant CYFI1-M151. The knockouts (CYFI1-M28 and CYFI1-M142) and deletion mutant (CYFI1-M61) are also devoid of two smaller peaks, that are assumed to be lolitrem A and E, respectively (Figure 17).

Table 5 Rates of infection, fungal biomass and alkaloid production

Strain	Fungal Type [1]	Number of plants/ association	Infection Rate[2] (%)	Lolitrem (ppm)	Ergovaline (ppm)	Peramine (ppm)
CYFI1M-28	KO	5	20	0	0.4 - 1.3	30 - 40
CYFI1M-61	Del	4	17	0	0.7 - 3.3	24 - 41
CYFI1M-142	KO	5	17	0	0.1 - 2.0	14 - 47
CYFI1M-151	Ectopic	5	17	4.4 - 16.7	0.5 - 1.2	21 - 55
FI1	Wt	4	22	6.2 - 12.8	0.8 - 1.5	31 - 66
Endophyte Free	NA	3	NA	0	0	0

[1]KO = *ltmM* knockout, Del = deletion mutant, Wt = Wildtype, NA = Not applicable.

[2]Infection rates were determined as a percentage of endophyte infected from the surviving plants. The infection rates are low but typical for the technique as the endophyte is inserted into young plants at a wound site.

Example 5 Construction and sequencing of Suppressive Subtractive Hybridisation Libraries

To identify additional genes involved in the lolitrem biosynthetic pathway an approach of EST sequencing from both *N. lolii in vitro* culture derived cDNA libraries and from subtracted plant derived cDNA libraries was adopted. ESTs within the libraries derived from *N. lolii* and with homology to genes from the paxilline biosynthetic pathway are good candidates for orthologous lolitrem biosynthetic genes. It was expected that some genes may be expressed in *in vitro* cultures but many may only be expressed *in planta* so the dual approach was taken. The *in vitro* culture libraries were derived from liquid cultures in both rich and minimal media to increase the chance of identifying ESTs that may only be expressed under starvation conditions and are described in example 6. The subtracted libraries were derived by constructing cDNA from both infected and uninfected perennial ryegrass plants and performing suppressive subtractive hybridization to enrich for fungal cDNAs.

Infected Plant Material

Perennial ryegrass genotypes are genetically complex due to the outbreeding nature of this species. To eliminate plant genotype effects and enable the comparison of infected and uninfected perennial ryegrass plants with identical genetic backgrounds cloned lines of infected Nui were cured of the fungus. The isogenic ryegrass lines infected or uninfected with N. lolii strain Lp19 were produced as described below. Lp19 is a endophyte from the AgResearch collection and it is known to produce Lolitrem B, Ergovaline and Peramine. Lp19 is an endophyte that has been isolated from its original parent plant and inoculated into the ryegrass cultivar Nui.

Positive and negative clones of the above material were produced by taking a positive plant and dividing the tillers up to produce a number of cloned plants. Some of the clones were then treated with a systemic fungicide to eliminate the endophyte.

This was done by striping tillers down and soaking in a 2g/L solution of Benlate (50% Benomyl w/w) for several hours then planting them in clean river sand saturated with the solution. Pots were watered to weight for several weeks such that the tillers were essentially immersed in fungicide for this period. Plants are potted into commercial potting mix and tillers assayed for endophyte presence. Endophyte free tillers were removed to new pots and tested periodically for endophyte presence to ensure that the fungus has been successfully eliminated. In this way we obtain E+ and E- cloned copies of an individual ryegrass genotype.

Plants were grown in the glasshouse in pots containing commercial potting mix. Plants were dissected in order to provide emerging immature leaf tissue and mature sheath tissue. Material was harvested and frozen immediately at -80C until needed.

Development of Suppressive Subtractive Hybridisation Libraries

RNA was extracted from the harvested plant tissues using the Triazol method (Invitrogen) following the manufacturers recommendations. Messenger RNA was purified from this using mRNA purification kits (Amersham) following the manufacturers recommendations. Messenger RNA (mRNA) was used in subsequent subtractive hybridisations using the Suppressive Subtractive Hybridisation (SSH) kit (Clontech) as per the manufacturer's instructions.

Subtractions were carried out in both a 'forward' and 'reverse' direction using 'tester' and 'driver' cDNAs as follows:

Tester equals cDNA from infected plants (Nle+).

Driver equals cDNA from uninfected plants (Nle-).

Plant line	Leaf tissue	Library
Nle+M	Mature	Up-regulated
Nle-M	Mature	Down-regulated
Nle+I	Immature	Up-regulated
Nle-I	Immature	Down-regulated

Subtractions were carried out using tester and driver from both immature and mature tissue and in both directions. Forward subtractions enrich for up-regulated genes and reverse subtractions enrich for down-regulated genes. After the subtraction procedure, cDNAs were ligated into the vector pCR-Topo2.1 (Invitrogen) and transformed into *E. coli* competent cells following the manufacturers recommendations. 1000 clones from each library were stored as glycerols in 96 well format.

Template preparation and Library sequencing

For sequencing template preparation PCR reactions were carried out in 384-well plates using the M13 forward (GTAAAACGACGGCCAG) and Reverse primers (CAGGAAACAGCTATGAC). A Biomek 2000 liquid handling robot was used to transfer 1 µl aliquots from each of 4 x 96-well plates containing overnight cultures into a conical bottomed 384-well plate (ABGen). PCR products were precipitated using 1 µl of 3M NaoAC (pH 6) and 15 µl of isopropanol and placed at -80°C for at least one hour before centrifugation at 4K for 1 hr (4°C). Pellets were washed with 20 µl of 70% ethanol and centrifuged for a further 30 min at 4K (4°C) before they were air dried and resuspended in 10 µl of sterile MQ water. Products were checked by running 1 µl samples on a 1% agarose gel (1X TAE).

Sequencing reactions were performed in conical bottomed 384-well plates (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). One µl of PCR product was added to 9 µl of sequencing mix (0.8 µl of 2

µM M13 Reverse primer; 0.5 µl Big Dye (Version 3); 3.5 µl ABI dilution buffer (400 mM Tris pH9; 10 mM $MgCl_2$) and 4.2 µl sterile MQ water) and the plate centrifuged briefly to collect the contents at the bottom of the wells. Cycle sequencing was performed using 40 cycles of 95°c for 20 sec, 50°C for 15 sec and 60°c for 1.5 min (iCycler, Bio-Rad, USA). Sequencing products were precipitated by the addition of 1 µl of 3M NaOAc (pH 4.6), 1 µl sterile MQ water and 23 µl non-denatured 95% ethanol, placed on the bench for 15 min (at RT) and centrifuged at 4K for 30 min (4°C). Immediately following centrifugation, plates were turned upside down on to several sheets of paper towels and centrifuged at 50 x *g* for 1 min to expel all liquid. Any remaining liquid was removed by briefly spinning the plate in a salad spinner and the pellets resuspended in 10 µl of HiDi™ formamide solution (Applied Biosystems). Sequencing was performed on the ABI 3100 (Applied Biosystems) using a 36 cm array.

Example 6. Construction of EST Database from *in vitro* Cultures

To identify additional genes involved in the lolitrem biosynthetic pathway an approach of EST sequencing from *N. lolii in vitro* culture derived cDNA libraries was adopted. ESTs within the libraries derived from *N. lolii* and with homology to genes from the paxilline biosynthetic pathway are good candidates for orthologous lolitrem biosynthetic genes. It was expected that some genes may be expressed in *in vitro* cultures but many may only be expressed *in planta* so an *in planta* approach is described in example 5. The *in vitro* culture libraries were derived from liquid cultures in both rich and minimal media to increase the chance of identifying ESTs that may only be expressed under starvation conditions.

Culture Conditions

N. lolli strain Lp19 was initially cultured on potato dextrose agar plates. Mycelia from the leading edge of colonies were removed and chopped up finely with a scalpel

blade before being transferred to 50 ml potato dextrose broth and incubated for 10 days at 25°C/200 rpm. Mycelia for RNA extraction were harvested under vacuum using a sterile buchner funnel containing two layers of Whatman 3 MM paper. Mycelia were washed three times with sterile MQ water. Dry weight was estimated from the wet weight.

To grow mycelia in minimal media, mycelia from *N. lolli* strain Lp19 cultures initially grown in complete medium for 14 days were harvested under vacuum using a sterile buchner funnel containing two layers of Whatman 3 MM paper. Mycelia were washed three times with sterile MQ water before transfer to the minimal medium. Two grams of mycelia was used to inoculate 50 ml of Blankenship MM (Blankenship et al. 2001) and the cultures incubated for 19 days at 25°C/200 rpm. Mycelia for RNA extraction were harvested under vacuum using a sterile buchner funnel containing two layers of Whatman 3 MM paper. Mycelia were washed three times with sterile MQ water. Dry weight was estimated from the wet weight.

Isolation of total RNA from cultures grown in complete medium

Mycelia were ground in liquid nitrogen using a sterile mortar and pestle and the RNA extracted using 1 ml Trizol® Reagent (Invitrogen) per 100 mg of ground mycelia. Samples were mixed well and frozen overnight at -20°C. The following day samples were thawed at RT on an orbital mixer (approx. 1 hr) and centrifuged at 12000 *x* g for 10 min (4°C) to remove polysaccharides. The supernatant was removed to a fresh tube and 0.2 ml of chloroform added per 1 ml Trizol reagent. Tubes were capped well and shaken vigorously by hand for 15 s and incubated at RT for 2 to 3min. Samples were centrifuged at 12000 *x* g for 15 min at 4°C and the supernatant removed with a pipette to a fresh tube. RNA was precipitated using a modified precipitation step that effectively precipitated the RNA while maintaining polysaccharides and proteoglycans in a soluble form. Essentially, 0.25 ml isopropanol was added to the supernatant followed by 0.25 ml of a high salt precipitation solution (0.8M sodium citrate and 1.2M

NaCl) per 1 ml of Trizol reagent used for the initial homogenization. The resulting solution was mixed well and the samples incubated at RT for 10 min. Samples were centrifuged at 12 000 *x* g for 10 min at 4°C and the resulting RNA pellet washed once with 75% ethanol (1 ml 75% ethanol per 1 ml Trizol). The sample was mixed by vortexing and centrifuged at 7500 *x* g for 5 min at 4°C.

The RNA pellet was briefly air dried for 5-10 min at RT and dissolved in 1 ml RNase free water (Invitrogen) with 1 µl Protector RNase (Roche) by passing the solution several times through a pipette tip and incubating for 10 min at 55-60°C. RNA purity and concentration were determined by spectrophotometry ($A_{260/280}$) and by running 3 µl and 6 µl aliquots (containing 1 µl of 10X MOPS running dye (0.2 M MOPS (pH7), 20 mM sodium acetate, 10 mM EDTA (pH8) in a total volume of 10 µl) on a 1% agarose gel in 1 X TAE buffer containing ethidium bromide (1 µg/ml). RNA was stored as 10 µl aliquots at -80°C.

Isolation of total RNA from cultures grown in minimal medium

Mycelia were ground in liquid nitrogen using a sterile mortar and pestle and the RNA extracted using 1 ml Trizol® Reagent (Invitrogen) per 100 mg of ground mycelia. Samples were mixed well and frozen overnight at -20°C. The following day samples were thawed at RT on an orbital mixer (approx. 1 hr) and 0.2 ml chloroform added per 1 ml of Trizol reagent. Samples were vigorously shaken by hand for 15 s and incubated at RT for 2-3 min. Samples were centrifuged at 12 000 *x* g for 15 min at 4°C and the upper aqueous phase removed to a fresh tube. RNA was precipitated using 0.5 ml isopropanol per 1 ml Trizol reagent used for the initial homogenization. Samples were incubated at RT for 10 min and centrifuged at 12 000 *x* g for 10 min at 4°C. The RNA pellet was washed using 1 ml 75% ethanol per 1 ml Trizol reagent used for the initial homogenization. Samples were mixed by vortexing and centrifuged at 7500 *x* g for 5 min at 4°C. The RNA pellet was briefly air dried for 5-10 min at RT and dissolved in 1 ml RNase free water (Invitrogen) with 1 µl Protector

RNase (Roche) by passing the solution several times through a pipette tip and incubating for 10 min at 55-60°C. RNA purity and concentration were determined by spectrophotometry ($A_{260/280}$) and by running 3 µl and 6 µl samples on a 1% agarose gel in 1 X TAE buffer containing ethidium bromide. RNA was stored as 10 µl aliquots at -80°C.

Purification of mRNA

mRNA was purified from total RNA using the mRNA Purification Kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Each Oligo (dT)-cellulose column had the capacity to bind approximately 25 µg of poly(A)$^+$RNA so, assuming that only 2% of the total RNA was polyadenylated, no more than 1.25 mg of total RNA was applied to each column. mRNA was subjected to two rounds of purification and the concentration determined by spectrophotometry ($A_{260/280}$). Aliquots were stored at -80°C.

First-strand cDNA synthesis using mRNA

Two µl of mRNA was combined with 1 µl SMART IV™ oligonucleotide and 1 µl CDS III/3' PCR primer in a sterile thin-walled 0.2 ml PCR tube (Bio-Rad). The contents were mixed, spun briefly in a microfuge and incubated at 72°C for 2 min. The tube was then cooled on ice for 2 min, spun briefly to collect the contents at the bottom of the tube and the following added:

2 µl 5X First-Strand Buffer

1 µl DTT (20 mM)

1 µl dNTP mix (10 mM)

1 µl PowerScript™ Reverse Transcriptase

Samples were mixed by gentle pipetting, briefly spun to collect the contents and incubated at 42°C for 1 hr (Bio-Rad iCycler). The tube was placed on ice to terminate first-strand synthesis, 1 µl of sodium hydroxide (25 mM) added and the tube incubated at 68°C for 30 min. A 3 µl aliquot was removed for cDNA amplification by Primer Extension PCR and the remaining first-strand cDNA stored at -20°C.

First-strand cDNA synthesis using total RNA

Three µl of freshly-prepared total RNA was combined with 1 µl SMART IV oligonucleotide and 1 µl CDS III/3' PCR primer in a sterile thin-walled 0.2 ml PCR tube (Bio-Rad). The contents were mixed, spun briefly in a microfuge and incubated at 72°C for 2 min. The tube was then cooled on ice for 2 min, spun briefly to collect the contents at the bottom of the tube and the following added:

2 µl 5X First-Strand Buffer

1 µl DTT (20 mM)

1 µl dNTP mix (10 mM)

1 µl PowerScript™ Reverse Transcriptase

Samples were mixed by gentle pipetting, briefly spun to collect the contents and incubated at 42°C for 1 hr (Bio-Rad iCycler). The tube was placed on ice to terminate first-strand synthesis and a 3 µl aliquot removed for cDNA amplification by Long Distance (LD) PCR. The remaining first-strand cDNA was stored at -20°C.

cDNA amplification by Primer Extension PCR

The following components were combined in a sterile 0.2 ml thin-walled PCR tube:

11 µl First Strand cDNA

71 µl sterile MQ water

10 µl 10X Advantage 2 PCR buffer

2 µl 50X dNTP mix

2 µl 5' PCR primer

2 µl CDS III/3' PCR primer

2 µl 10X Advantage 2 Polymerase mix

Samples were mixed, briefly spun to collect the contents and amplified by PCR (72°C for 10 min, 95°C for 20 s and 3cycles of 95°C for 5 s, 68°C for 8 min) using the Bio-Rad iCycler. A 10 µl sample was analysed on a 1.0% agarose gel (1X TAE) alongside 0.1 µg of a 1 kb plus DNA size marker (Invitrogen). The ds cDNA either underwent subsequent proteinase K and *Sfi* I digestions or was stored at -20°C until further use.

cDNA amplification by LD PCR

The following components were combined in a sterile 0.2 ml thin-walled PCR tube (Bio-Rad):

3 µl First-Strand cDNA

79 µl sterile MQ water

10 µl Advantage 2 PCR buffer

2 µl 50X dNTP mix

2 µl 5' PCR Primer

2 µl CDS III/3' PCR Primer

2 µl 50X Advantage 2 Polymerase Mix

Samples were mixed by gently flicking the tube, briefly spun to collect the contents and amplified by PCR (95°C for 30 s and 26 cycles of 95°C for 15 s, 68°C for 6 min) using the Bio-Rad iCycler. The ds cDNA either underwent subsequent proteinase K and *Sfi* I digestions or was stored at -20°C until further use.

Four µl of Proteinase K (20 µg/µl) and 5 µl of sterile MQ water were added to 90 µl of amplified ds cDNA, mixed and incubated at 45°C for 20 min. The reaction was cleaned up using the Qiagen PCR Purification Kit as per the manufacturer's instructions and the cDNA eluted from the column in a total volume of 50 µl.

The following components were added to a fresh 0.2 ml thin-walled PCR tube:

50 µl	cDNA (proteinase K treated)
29 µl	sterile MQ water
10 µl	10X *Sfi* I buffer
10 µl	*Sfi* I restriction enzyme
1 µl	100X BSA

Samples were mixed well and incubated at 50°C for 2 hr.

Following *Sfi* I digestion, 2 µl of a 1% xylene cyanol solution was added to the tube and the sample mixed well. Sixteen sterile 1.5 ml tubes were labelled and arranged in a rack in order. A CHROMA SPIN-400 column (Clontech) was prepared as per the manufacturer's instructions and the mixture of Sfi I-digested cDNA and xylene cyanol dye carefully applied to the top centre surface of the column matrix. Once the sample was fully absorbed into the matrix, 100 µl of column buffer was also applied to the column and the buffer allowed to drain from the column until there was no liquid remaining above the resin. At this point, the dye layer was several mm into the column.

The rack containing the 1.5 ml collection tubes was placed so that the first tube was directly underneath the column outlet. 600 µl of column buffer was added to the column and single-drop fractions (approximately 35 µl per tube) collected in the labelled tubes. The profile of each fraction was checked by analysing 10 µl samples alongside 0.1 µg of a 1 kb plus DNA standard (Invitrogen) on a 1.1% agarose gel (1X TAE; 150V; 10 min). The gel was stained with ethidium bromide for 15 min, destained in water for 1.5 hr and the peak fractions determined by visualizing the intensity of the bands under UV. The first 3 fractions containing cDNA were collected and pooled. Samples were cleaned up using an Amicon-30 unit (Millipore). The unit was washed twice with sterile MQ water before use as per the manufacturer's instructions. The pooled fractions were applied to the unit and concentrated to 7 µl by centrifugation at 14 000g for 20 min at room temperature. The *Sfi* I-digested cDNA was either stored at -20°C or used immediately in the ligation reaction.

Ligation of Sfi I-digested cDNA to the λTriplEx2 Vector and library packaging

Ligations were optimized using three different ratios of cDNA to phage vector following the manufacturers recommendations. Samples were mixed gently, centrifuged briefly to bring the contents to the bottom of the tube and incubated overnight at 16°C. Ligations (cDNA/λTriplEx2 Vector) were heat inactivated at 65°C for 15 min. Packaging reactions (50 µl) were thawed at room temperature and placed on ice. Half of the packaging extract (25 µl) was immediately transferred to a second ice-cold 1.5 ml tube. The entire ligation (7 µl) was added to 25 µl of packaging extract, mixed gently with a pipette and incubated at 30°C for 90 min. At the end of this incubation, the remaining 25 µl of packaging extract was added to the sample and the reaction incubated for a further 90 min at 30°C. Five hundred µl of 1X Lambda dilution buffer (100 mM NaCl, 10 mM $MgSO_4.7H_2O$, 35 mM Tris-HCl (pH 7.5), 0.01% gelatin) was added to the sample and mixed by gentle vortexing. Chloroform (25 µl) was also added to prevent bacterial contamination. Packaged

libraries were titered following the manufacturers recommendations and stored at 4°C for up to one month.

Library Amplification

A single, well-isolated colony of XL1-Blue was picked from the primary working plate and used to inoculate 15 ml of LB broth containing $MgSO_4$ (10 mM) and maltose (0.2%). Cultures were incubated at 37°C overnight with shaking (140 rpm). Cells were harvested the following day by centrifuging the culture at 5K for 5 min. The supernatant was removed by decanting and the pellet resuspended in 7.5 ml of 10 mM $MgSO_4$. Enough phage to yield 6-7 x 10^4 plaques per 150 mm plate was added to each of 10 tubes containing 500 µl of overnight XL1-Blue culture in a sterile 1.5 ml tube. Phage were allowed to adsorb to the *E. coli* cells by incubating in a 37°C water bath for 15 min before adding 4.5 ml of melted (45°C) LB top agar containing $MgSO_4$ (10 mM) and maltose (0.2%). Samples were quickly mixed by gentle vortexing and immediately poured on to prewarmed (37°C) 150 mm LB agar plates containing $MgSO_4$ (10 mM). Plates were cooled for 10 min at room temperature to allow the top agar to harden and incubated at 37 °C for 10.5 hr. Phage were eluted by adding 12.5 ml of 1X Lambda dilution buffer to each plate and the plates stored overnight at 4°C. The following day, the plates were shaken (~50 rpm) at room temperature for 1 hr and the phage lysates poured into a sterile beaker. Intact cells were lysed by adding 10 ml of chloroform and the phage lysate cleared of cell debris by centrifuging at 5 000 *x* g for 10 min in sterile 50 ml polypropylene tubes. The supernatant was collected and stored at 4°C in sterile universals. For long-term storage, 1 ml aliquots were made containing DMSO to a final concentration of 7% and frozen at -80°C.

Converting λTriplEx2 to pTriplEx

The bacterial host strain *E. coli* BM25.8 (*supE*44, thi Δ(lac-proAB) *relA*1, [F' *lacI*^{q}ZΔ*M15*, *proAB*$^+$, *traD*36], *hsdR*(r_{k12}-m_{k12}-), (*kan*^{R})P1 (*cam*^{R}) *λimm*434) was

supplied as a component of the SMART cDNA Library Construction Kit (Clontech) and stored at -80°C. For large-scale library conversion a single, well-isolated colony of *E. coli* BM25.8 was picked from the primary working plate and used to inoculate 10 ml of LB broth. Cultures were incubated at 31°C overnight with shaking (150 rpm). The following day, $MgCl_2$ (10 mM) was added to the overnight culture of BM25.8. In a sterile 15 ml tube, 200 µl of overnight culture was mixed with 2 x 10^6 pfu/ml of amplified λTriplEx2 cDNA library and incubated for 1 hr at 31°C (without shaking). After the incubation was complete, 500 µl of LB broth was added and the sample incubated for a further 1 hr at 31°C with shaking (190 rpm). At this point, conversion of the library to plasmid form was complete. The converted cDNA library was diluted 1:100 in LB broth and aliquots (10 µl, 100 µl) were spread on to LB agar plates containing carbenicillin (50 µg/ml). Plates were incubated overnight at 31°C and the colonies picked for further analysis. The remaining converted library was stored as 1 ml aliquots containing glycerol (to a final concentration of 30%) at -80°C.

PCR analysis

Individual colonies from converted libraries were inoculated into 100 µl of LB broth containing carbenicillin (50 µg/ml) in round bottomed 96-well plates (Nunc). Plates were incubated overnight at 37°C. Aliquots of 1 µl of each overnight culture were PCR amplified in a total volume of 15 µl using ptriplex2FORWARD (5'-AAGCGCGCCATTGTGTTGGTACCC-3') and ptriplex2REVERSE (5'-CGGCCGCATGCATAAGCTTGCTCG-3') as primers (present in the pTriplEx vector arms) (Kohler *et al.*, 2003). The PCR included 95°C for 3 min, 95°C for 60 s, 60°C for 30 s, 72°C for 3 min for 30 cycles and a final extension of 72°C for 15 min (iCycler, Bio-Rad, USA). One µl of each reaction was analysed on a 1% agarose gel alongside 0.25 µg of a 1 kb plus DNA standard (Invitrogen) and stained with ethidium bromide to determine the size and quality of the PCR products.

For sequencing template preparation PCR reactions were carried out in 384-well plates. The Biomek 2000 liquid handling robot was used to transfer 1 µl aliquots from each of 4 x 96-well plates containing overnight cultures into a conical bottomed 384-well plate (ABGen). PCR products were precipitated using 1 µl of 3M NaoAC (pH 6) and 15 µl of isopropanol and placed at -80°C for at least one hour before centrifugation at 4K for 1 hr (4°C). Pellets were washed with 20 µl of 70% ethanol and centrifuged for a further 30 min at 4K (4°C) before they were air dried and resuspended in 10 µl of sterile MQ water. Products were checked by running 1 µl samples on a 1% agarose gel (1X TAE) and further diluted either 1:5 (minimal medium cDNA library) or 1:1 (complete medium cDNA library) in sterile MQ water before sequencing.

Sequencing Reactions

Sequencing reactions were performed in conical bottomed 384-well plates (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). One µl of PCR product was added to 9 µl of sequencing mix (0.8 µl of 2 µM ptriplex2FORWARD primer; 0.5 µl Big Dye (Version 3); 3.5 µl ABI dilution buffer (400 mM Tris pH9; 10 mM $MgCl_2$) and 4.2 µl sterile MQ water) and the plate centrifuged briefly to collect the contents at the bottom of the wells. Cycle sequencing was performed using 40 cycles of 95°c for 20 sec, 50°C for 15 sec and 60°c for 1.5 min (iCycler, Bio-Rad, USA). Sequencing products were precipitated by the addition of 1 µl of 3M NaOAc (pH 4.6), 1 µl sterile MQ water and 23 µl non-denatured 95% ethanol, placed on the bench for 15 min (at RT) and centrifuged at 4K for 30 min (4°C). Immediately following centrifugation, plates were turned upside down on to several sheets of paper towels and centrifuged at 50 x *g* for 1 min to expel all liquid. Any remaining liquid was removed by briefly spinning the plate in a salad spinner and the pellets resuspended in 10 µl of HiDi™ formamide solution (Applied Biosystems).

Sequencing was performed on the ABI 3730 (Applied Biosystems) using a 50 cm array.

Example 7. Identification of ESTs Encoding Putative Lolitrem Biosynthetic Genes from EST Sequence Databases

A sequence database was developed containing the 4000 EST sequences derived from the SSH libraries and 6500 ESTs derived from *in vitro* culture libraries. The database was searched using the BLAST algorithm. Nucleotide sequences were blasted using the BlastX algorithm against the SwissProt database. ESTs with homology to paxilline biosynthetic genes are listed in Table 6. All paxilline orthologs were identified in the Nle+M library.

Table 6. Detail of ESTs with Homology to Paxilline Biosynthetic Genes

EST	Length (bp)	Function	Paxilline Homolog	Blast Score
E07	353	dimethylallyltryptophan (DMAT) synthase	paxD	5e-02
DMAT Johnson1	532	dimethylallyltryptophan (DMAT) synthase	paxD	
N17	413	Cytochrome P450 monooxygenase	-	2e-09
G13	335	Cytochrome P450 monooxygenase	paxP	3e-07
J15	639	Cytochrome P450 monooxygenase	paxP	8e-34

Example 8. Predicted Genes in Lolitrem Gene Cluster and Isolation of two additional ltm gene clusters

Isolation of Lolitrem Biosynthetic Genes

We describe in examples 2 and 3 the molecular cloning and genetic analysis of a set of genes from *N. lolii* and *E. festucae* that are proposed to be involved in the biosynthesis of lolitrem and closely related indole-diterpenes. This is the second indole-diterpene gene cluster to be cloned from a filamentous fungus, the other being a cluster of genes from *P. paxilli* required for paxilline biosynthesis (Young et al. 2001).

A comparison with the paxilline biosynthesis cluster identifies five functional orthologues, *ltmG*, *ltmM*, *ltmP*, *ltmQ* and *ltmD*. In addition we have identified two P450 genes, *ltmJ* and *ltmK*, that may also play a role in indole-diterpene biosynthesis in this group of fungi. Three of these genes, *ltmG*, *ltmM* and *ltmK* form a tightly linked cluster.

The first of these genes, *ltmG*, is clearly identifiable as a GGPP synthase, and is presumed to catalyse the first step in the biosynthesis of lolitrems i.e. the synthesis of GGPP. Interestingly, the two fungal species in which diterpene gene clusters have been analysed, have two copies of GGPP synthase, one proposed to be involved in primary metabolism and one specifically recruited for secondary metabolism (Tudzynski and Hölter 1998; Young et al. 2001). *N. lolii* and *E. festucae* also have two copies of a GGPP synthase. One copy is proposed to be required for primary metabolism and the second copy (*ltmG*) is proposed to be specifically required for indole-diterpene biosynthesis.

Deletions of *paxM* and *paxC* in *P. paxilli* result in mutants with a paxilline-negative phenotype. To date no identifiable indole-diterpene intermediates have been identified in these strains, suggesting that these genes are involved in very early steps in the pathway. Our working model is that PaxM and PaxC are required to catalyse the epoxidation and cyclisation of GGPP and addition of indole-3-glycerol to form the first stable indole-diterpene, possibly paspaline (Parker and Scott 2004). By analogy we propose that LtmM catalyses the same early reaction in lolitrem biosynthesis. In support of this hypothesis we were able to demonstrate that *ltmM* is required for lolitrem biosynthesis by making a targeted deletion of this gene. Mutants deleted in this gene were unable to synthesize lolitrem B in artificial symbiota with perennial ryegrass. An *N. lolii* orthologue of *paxC*, is yet to be identified, but is predicted to also be essential for lolitrem biosynthesis.

Other genes identified as being necessary for paxilline biosynthesis are *paxP* and *paxQ*; which encode cytochrome P450 enzymes. Targeted deletion of *paxP* and *paxQ* results in strains that accumulate paspaline and 13-desoxypaxilline, respectively (McMillan et al. 2003). These results suggest that PaxP is required for demethylation of C-12 of paspaline, and possibly hydroxylation of C-10, and PaxQ is required for hydroxylation of C-13, using either PC-M6 or 13-desoxypaxilline as substrates (Figure18). Analysis of the structure of lolitrem B (Figure1) suggests that similar modifications are required to the paspaline skeleton (Figure18) to generate lolitrem B. Orthologues of *paxP* and *paxQ* were identified in an EST library generated with template from suppression subtractive hybridization. A further enzyme predicted to be required for lolitrem B biosynthesis is a prenyl transferase to prenylate positions 20 and 21 of the indole ring. A candidate gene for one or both of these prenylations is *ltmD*, given that the paralogue, *dmaW*, prenylates position 20, as the first committed step in ergot alkaloid biosynthesis (Wang et al. 2004). One or two additional cytochrome P450 enzymes are predicted to be required for further oxidation and closure of ring A of lolitrem B. Candidates for these functions include *ltmJ* and *ltmK*. At least two additional enzymes are required to form an epoxide between C-11 and C-12 of paspaline, and prenylate ring H of lolitrem B. These would be predicted to be an FAD-dependent monooxygenase and a prenyl transferase, respectively. We have yet to identify these genes.

In summary, we predict that up to ten genes are required for the biosynthesis of lolitrem B. Candidate genes identified to date include *ltmG*, *ltmM*, *ltmK*, *ltmP*, *ltmQ*, *ltmD* and *ltmJ*. Deletion analysis has confirmed that at least *ltmM* is required for lolitrem B biosynthesis. Further genetic analysis of the genes identified here and adjacent genes will help elucidate the pathway for lolitrem biosynthesis. A comparison with the steps required for paxilline biosynthesis in *P. paxilli* will elucidate the basic biochemistry and genetics of this important group of secondary metabolites. In Example 7 we describe the isolation of ESTs that may correspond to *ltmP, ltmD* and

ltmJ. In this example we describe a method for isolation of genomics regions containing the additional predicted *ltm* genes.

Isolation of Additional ltm Genes

The presence of the retrotransposon platforms and the instability of cloned fragments of the regions directly flanking the *ltmG, ltmM* and *ltmK* cluster made it difficult to isolate additional flanking sequences. Therefore we used EST sequences with homology to the *paxP* gene to isolate an orthologue (*ltmP*). The EST sequences G13, J15 and N17 isolated from endophyte infected ryegrass in Example 7 showed significant homology to *paxP* (Table 6). In this example we demonstrated that these ESTs corresponded to *ltmP* and were linked to the cluster containing *ltmG*, *ltmM* and *ltmK*.

The EST sequences G13, J15 and N17 did not align to the cluster containing *ltmG*, *ltmM* and *ltmK* suggesting they were unique. Primers were then designed to regions that were highly conserved to *paxP* with a consideration on the placement of possible conserved introns between the *ltm* and *pax* genes.

EST sequences with BLASTX matches to *paxP* aligned into three independent contigs (Figure26). Contig 1 contained EST sequence J15, contig 2 contained EST sequences G13, and contig 3 contained EST sequence N17. PCR was performed to test whether these three contigs were part of a single cytochrome P450 monooxygenase gene or were in fact multiple genes. Amplification of Lp19 genomic DNA with primers lol192 and lol195 linked contigs 1 and 2 and therefore these two contigs are a part of the same fungal cytochrome P450 monooxygenase gene subsequently named *ltmP*. The PCR fragment generated from Lp19 genomic DNA with primers lol192 and lol195 was sequenced and compared to the EST data for confirmation of the intron. Contig 3 contained the primer binding site for primer lol194 and this primer would not amplify a PCR product from Lp19 genomic DNA when

paired with primer lol192. This contig was therefore considered an independent cytochrome P450 monooxygenase fragment and was subsequently named *ltmJ*. Primers, lol205 and lol206, were designed to the contig sequence of *ltmJ*. These primers amplified a 242 bp fragment from Lp19 genomic DNA and confirmed that *ltmJ* was of fungal origin.

Table 9 Primers used in this example and not listed in table 2

Primer name	Sequence 5'→3'	Used for
lol191	CCAAAGGAGGTTTTGAATGTA	*ltmP* PCR/probe
lol192	TTGGATGAGCTCAATCATGC	*ltmP* PCR/probe/RT-PCR
lol194	GAACTCGTAGCGCAGGAGCA	*ltmJ* PCR
lol195	TTCTCTTCGGAGGCTCTCCT	*ltmP* PCR
lol196	TGGACATGGATCTGATTGTC	*ltmP* probe
lol198	TGTAGCACGGGTAGCTAGAT	*ltmP* probe
lol199	TTGCGCATCGTACGCTAGGA	IPCR
lol202	GGATGAAGAAAATCCACGAG	IPCR
lol203	AGACGATCTGTTAGGCCGAT	IPCR
lol205	CCAAGCATCGATTTGTCACC	*ltmJ* PCR/probe
lol206	AATCTGATCGCCATCTTTGC	*ltmJ* PCR/probe
lol209	GAATAGCTCAAGACTCAGAA	IPCR
lol210	AAGCTGGCTGTTAAAGGGTC	IPCR
lol211	TATTAGGGAGCGAACTTCAC	IPCR
lol213	AAGAGGGCCGCAATTTCGAT	IPCR
lol222	GCGTGCAACATTAACATTCTC	IPCR
lol235	ATTCCACCATGGCATCTGGAGCATGGCTC G	*ltmC* complenetation
lol236	CTTAAGCGAATTCTACCTTGTGGGTC	*ltmC* probe/complementation
lol341	TTCCGCTTCCGAGTAGACTC	*ltmE* PCR/RT-PCR/probe
lol356	CCGAGTTTGATGACCTGCTG	*ltmE* PCR/RT-PCR/probe
SP6	CCATTTAGGTGACACTATAG	Seq
T1.1	GAGAAAATGCGTGAGATTGT	*tub2* probe/RT-PCR
T1.2	CTGGTCAACCAGCTCAGCAC	*tub2* probe/RT-PCR

The *ltmJ* fragment hybridised to the lolitrem producing strains Lp19 and Fl1 (Figure 47). This fragment hybridised to a ~18 kb Lp19 *Sst*I fragment, a band of the same size as seen with the *ltmP* probes suggesting linkage of *ltmJ* to *ltmP*. The presence of the three EST fragments, *ltmC, ltmP* and *ltmJ*, correlated with strains known to produce indole-diterpenes. None of the fragments hybridised to genomic digests of E8, a lolitrem non-producing strain. This pattern of hybridisation was used to identify the previous *ltm* cluster containing *ltmG*, *ltmM* and *ltmK* and therefore complete sequence surrounding the genes *ltmC, ltmP* and *ltmJ* was obtained.

ltm cluster 2

Initially the complete *ltmC* and *ltmP* genes were sequenced from Lp19 using fragments generated by IPCR with the restriction enzymes *Eco*RI, *Eco*RV and *Hin*dIII (Figure28). The complete *ltmC* gene was amplified using IPCR with Lp19 *Hin*dIII digested then self-ligated genomic DNA and primers, lol202 and lol203, that were designed to the previously obtained *ltmC* sequence. The sequence was extended using IPCR with Lp19 *Eco*RI digested then self-ligated genomic DNA and primers, lol213 and lol209. The sequence of the complete *ltmP* gene was generated using IPCR with Lp19 *Hin*dIII digested then self-ligated genomic DNA using two primer sets of lol198 and lol199, and lol210 and lol211. The sequence was extended further by IPCR using Lp19 *Eco*RV digested then self-ligated genomic DNA with primers lol192 and lol222. Each IPCR fragment was cloned into pGEM-T easy (Promega) and sequenced with primers that were *ltmC* or *ltmP* sequence specific or with primers Sp6 and T7.

A Lp19 λGEM-12 genomic library was screened with *ltmP*. Lp19 λGEM-12 genomic library filters were screened with the *ltmP* fragment, amplified with lol191 and lol192, which resulted in the isolation of 25 positive clones. The average insert size of the 35-lambda clones was approximately 13 kb. DNA isolated from the lambda clones was digested with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, and *Sst*I then hybridised with the *ltmP* fragment to determine clones for sequencing. Lambda clones of interest were sequenced with primers Sp6 and T7 that anneal to the lambda arms and then with sequence specific primers. To facilitate sequencing, fragments from some lambda clones were cloned into the pUC118 vector and sequenced with the forward and reverse primers. A physical map of the overlapping lambda clones (Figure 27) was determined based on DNA sequence analysis and the hybridisation data from both the lambda clones and the genomic DNA (Figure27). Data generated from the physical map of the lambda clones showed that the following; λCY300,

λCY307, λCY312, λCY313, λCY315, λCY316, λCY319 and λCY350, contained sequences that were inconsistent with the Lp19 genomic map and therefore these sequences were not analysed further.

Nucleotide sequence generated from sequencing lambda clones isolated from the hybridisation with *ltmP* covered 23.8 kb. Sequence analysis of this region using BLAST algorithms identified three additional genes with significant similarities to *pax* genes from *P. paxilli* and therefore formed a second gene cluster called *ltm* cluster 2 (Figure27). The nucleotide sequence of *N. lolii* ltm cluster 2 is shown in Figure 28. These genes included, *ltmC*, *ltmP*, *ltmQ*, *ltmD*, and *ltm25*, orthologues of *paxC*, a prenyl transferase, *paxP* and *paxQ*, cytochrome P450 monooxygenases, *paxD*, a prenyl transferase (a dimethylallyl tryptophan synthase - like) and *sec25*, a gene of unknown function, respectively (Figure 27). The *ltmJ* gene was not contained within this sequenced region. The individual sequence analysis of the five genes contained in *ltm* cluster 2 is explained below and in Tables 7 and 8.

Table 73.11 The *ltm* genes from clusters 2 and 3, intron analysis and comparisons to database sequences

Gene	Putative function	Cluster	size (aa)	kDa	Intron				Top Database match[1]	Species	E value	Reference
					No.	phase	size	5'...3' Splice sites				
ltm25	Unknown	2	221	24.4	1	2	76	GTAAGT...CAG	sec25[2]	*P. paxilli*	2e-59	
ltmC	Prenyl transferase	2	345	39	1	2	77	GTATGT...TAG	paxC	*P. paxilli*	1e-59	Young et al 2001
ltmD	Prenyl transferase	2	439	49	1	2	74	GTAAGA...CAG	AN8514	*A. nidulans*	1e-46	
	(*dmaW*-like)				1	2	102	GTAAGT...TAG				
ltmQ	P450 monooxygenase	2	537	61.4	1	0	59	GTTTGA...AAG	paxQ	*P. paxilli*	1e-105	Young et al 2001
					2	0	61	GTTTGT...TAG				
					3	0	59	GTAAGT...CAG				
					4	2	60	GTAAGC...TAG				
					5	0	52	GTATGG...TAG				
					6	0	53	GTATAT...TAG				
					7	1	56	GTATAA...CAG				
ltmP	P450 monooxygenase	2	498	57	1	0	59	GTGTTC...CAG	paxP	*P. paxilli*	1e-102	Young et al 2001
					2	1	49	GTAAGT...CAG				

					3	1	60	GTATGT...GAG				
					4	1	57	GTAAGG...AAG				
					5	0	107	GTATGT...TAG				
ltmJ	P450 monooxygenase	3	525	60.4	1	0	63	GTGAGC...CAG	AN1598	*A. nidulans*	4e-81	
					2	0	67	GTGAGG...TAG				
					3	0	49	GTAAGG...AAG				
					4	1	69	GTATGT...TAG				
					5	1	61	GTAAGA...CAG				
					6	1	58	GTAAGT...TAG				
ltmE	Prenyl transferase fusion	3	788	87.5	1	2	67	GTACGT...AAG	paxC[3]	*P. paxilli*	3e-60	Young et al 2001
	(paxC-paxD)				2	2	56	GTGAGT...CAG	AN8514[3]	*A. nidulans*	7e-56	
					3	2	54	GTACGT...TAG				
chsV	Chitin synthase (class V)		1861	206	1		82	GTAGGT...TAG	AAF04279	*Blumeria graminis*	0.0	
					2		69	GTAAGT...CAG				

[1] If the top match was to a *pax* gene, the gene name was used otherwise the gene is indicated by the accession or gene number.
[2] The top match was determined using the tBLASTn algorithm as the *sec25* gene is currently not annotated

PCT/NZ2004/000333
Received 4 October 2005

[3] This gene is a hybrid containing a *paxC*-like domain and a *paxD*-domain

The nucleotide sequence analysis of the complete *ltmC* gene showed that it contains one intron (Figure 27 and 29) and encodes a polypepetide of 345 amino acids. LtmC is classified as a prenyl transferase as it contains the five conserved domains found in other prenyl transferases (**Chen et al, 1994**). FastA analysis showed that LtmC was more similar to AtmC from *A. flavus* than PaxC from *P. paxilli*. The single intron found in *ltmC* was conserved with placement and phase with the second of the two conserved introns found in *P. paxilli paxC* (Young et al, 2001) and *A. flavus atmC* (Zhang et al, 2004).

Table 8 Sequence identity of the *ltm* genes to their *pax* and *atm* homologues

gene	Homologue	*Species*	% Identity	E-value	Analysis programme
ltmG	*ggs1*	*P. paxilli*	54.1	1e-90	FASTA/BLASTP
	paxG	*P. paxilli*	52.6		FASTA
	paxC	*P. paxilli*	31.5		FASTA
	paxD	*P. paxilli*	22.2		FASTA
	atmG	*A. flavus*	59.4	e-101	FASTA/BLASTP
	atmC	*A. flavus*	30.1		FASTA
	ltmC	*N. lolii*	28.4		FASTA
	ltmD	*N. lolii*	28.4		FASTA
	ltmE	*N. lolii*	31.5		FASTA
ltmM	*paxM*	*P. paxilli*	41	7e-96	BLASTP
	atmM	*A. flavus*	42.2	e-100	BLASTP
ltmK	*paxP*	*P. paxilli*	31.3	7e-63	FASTA/BLASTP
	paxQ	*P. paxilli*	23.4		FASTA
	ltmJ	*N. lolii*	36.8		FASTA
	ltmP	*N. lolii*	28.6		FASTA
	ltmQ	*N. lolii*	25.3		FASTA
ltm25	*sec25*	*P. paxilli*	53.8	2e-59	tBLASTn
	FG04594	*Fusarium graminearum*		4e-46	BLASTP
ltmC	*paxC*	*P. paxilli*	43.3	1e-59	FASTA/BLASTP
	paxG	*P. paxilli*	28.4		FASTA
	atmC	*A. flavus*	47.7	2e-68	FASTA/BLASTP
	atmG	*A. flavus*	28.1		FASTA
	ltmE	*N. lolii*	55.8		FASTA
	ltmG	*N. lolii*	28.4		FASTA
ltmD	*paxD*	*P. paxilli*	24.2		FASTA
	ltmE	*N. lolii*	37.1		FASTA

	ltmG	*N. lolii*	24.2		FASTA
	dmaW	*Neotyphodium* LpTG-2	22.5		FASTA
	AN8514	*Aspergillus nidulans*		1e-46	BLASTP
ltmP	*paxP*	*P. paxilli*	41.3	e-102	FASTA/BLASTP
	paxQ	*P. paxilli*	24.4		FASTA
	ltmJ	*N. lolii*	25		FASTA
	ltmK	*N. lolii*	29.2		FASTA
	ltmQ	*N. lolii*	24.5		FASTA
ltmQ	*paxQ*	*P. paxilli*	38.1	e-105	FASTA/BLASTP
	paxP	*P. paxilli*	28.7		FASTA
	ltmJ	*N. lolii*	22.2		FASTA
	ltmK	*N. lolii*	25.3		FASTA
	ltmP	*N. lolii*	24.1		FASTA
ltmJ	*paxP*	*P. paxilli*	29.2	1e-49	FASTA/BLASTP
	paxQ	*P. paxilli*	23.7		FASTA
	ltmK	*N. lolii*	36.8		FASTA
	ltmP	*N. lolii*	25		FASTA
	ltmQ	*N. lolii*	21.9		FASTA
	AN1598	*A. nidulans*		4e-81	BLASTP
ltmE	*paxC*	*P. paxilli*	43.1	3e-60	FASTA/BLASTP
	atmC	*A. flavus*	49.5	7e-71	FASTA/BLASTP
	ltmC	*N. lolii*	55.8		FASTA
	ltmD	*N. lolii*	37.1		FASTA
	ltmG	*N. lolii*	32.8		FASTA
	AN8514	*A. nidulans*		7e-56	BLASTP

Sequence analysis of the complete *ltmP* gene, initially identified from EST sequences, showed that it contained five introns (Figure 27 and 31) and encodes a polypeptide of 498 amino acids (Figure 32). LtmP is classified as a cytochrome P450 monooxygenase based on database matches. The placement and phase of four introns, 1, 2, 3 and 4, are conserved with the *paxP* introns, 1, 3, 4 and 5 (**Young et al, 2001**); and three introns 1, 3, and 4, conserved with *ltmK* introns, 1, 4, and 7. LtmP is more similar to PaxP than to PaxQ or ltmK.

Adjacent to *ltmP* is *ltmQ,* a cytochrome P450 monooxygenase gene (Figure 28 and 33). The best database match to *ltmQ* is that of *paxQ* from *P. paxilli* and FastA analysis confirmed that LtmQ is more similar to PaxQ than to PaxP (Table 7). The nucleotide sequence of *N. lolii ltm* Q and deduced amino acid sequence of *ltm*Q polypeptide shown in Figure 33 and 34 respectively. The *ltmQ* gene contains 7

introns (Figure28 and 31) of which six, introns 2 to 7, are conserved in placement and phase with *paxQ* introns 2, 3, 5, 6, 7 and 8. It appears from ClustalW alignments of the *ltm* and *pax* P450 monooxygenases that *ltmQ* has lost a conserved intron, that is present in the remaining five sequences shown in the alignment, corresponding in position between the current *ltmQ* introns 3 and 4. The intron boundaries of *ltmQ* were confirmed by sequence comparison of RT-PCR products amplified using cDNA from endophyte infected plant material and gene specific primers, to the Lp19 genomic region.

The *ltm25* gene has no predicted function and had a best BLASTP match to an uncharacterised gene from *F. graminearum* FG04594, (accession number EAA72208). Using the tBLASTN algorithm against the public databases the best match was to *sec25*, a gene recently identified within the *P. paxilli pax* cluster, but as yet not publicly annotated (Monahan and Scott, unpublished). The *ltm25* gene has one intron (Figure 27 and 31) that is conserved in placement and phase with the *sec25* gene from *P. paxilli*. The nucleotide sequence of *N. lolii ltm* Q and deduced amino acid sequence of *ltm*Q polypeptide shown in Figure 35 and 36 respectively.

The *ltmD* gene, had a best BLASTP match to *A. nidulans* AN8514. The nucleotide sequence of *N. lolii ltm* D and deduced amino acid sequence of *ltm*Q polypeptide shown in Figure 37 and 38 respectively. FastA analysis was used to compare LtmD to DmaW from Lp1 a *Neotyphodium* LpTG-2 and PaxD from *P. paxilli* as the publicly available *paxD* sequence is not complete (Table 7). This data showed that LtmD is more similar to PaxD than to the DmaW from *Neotyphodium* LpTG-2. The *ltmD* gene contains two introns of which the placement and phase of intron 2 is conserved with *paxD*.

The predicted introns of the five genes contained in *ltm* cluster 2 were confirmed by sequence comparison of cDNA sequences, generated by RT-PCR using cDNA from endophyte infected plant material, with the genomic sequence. A summary of the

intron numbers, intron splice sites and predicted molecular mass in kDa of each gene is shown in Table 8.

Flanking *ltm25* is a Rua long terminal repeat (Rua4) and degenerate retrotransposon sequence (Figure 27). Upstream from *ltmP* is an AT-rich region that was devoid of obvious open reading frames and no genes were evident from sequence analysis using BLAST searches. Southern analysis with a fragment from this region to *Eco*RI, *Hin*dIII and *Sst*I digested DNA showed that this sequence is present in the lolitrem producing strains Lp19 and Fl1 but absent from the non-producer E8. Based on Southern analysis there are predicted to be ~3 - 5 copies of this sequence contained within the Lp19 and Fl1 genomes (data not shown). The presence of AT rich sequences adjacent to *ltm25* and *ltmP* suggested that no additional genes are present at this locus thereby defining the boundaries of *ltm* cluster 2.

ltm Cluster 3

The sequence surrounding *ltmJ*, a cytochrome P450 monooxygenase initially identified from EST sequence N17, was isolated from the Lp19 λGEM-12 genomic library hybridised with the *ltmJ* fragment, amplified with primers lol205 and lol206. This hybridisation resulted in the isolation of 22 positive clones. Fifteen clones were digested with *Hin*dIII or *Bam*HI and hybridised with the *ltmJ* fragment to determine clones of interest. Comparison of the restriction enzyme digests and sequencing of these clones, with primers, SP6 and T7, that anneal to the lambda arms, showed that only two identical clones, λCY324 and λCY344, had the correct genomic arrangement based on Southern and PCR analysis. Figure 39 shows a physical map of the cluster 3 locus. Other lambda clones were rearranged and/or contained unrelated sequences.

Sequence analysis of λCY346 and λCY324 identified from *ltmP* and *ltmJ* hybridisations respectively, were shown to overlap, linking *ltm* clusters 2 and 3 with a

16-kb AT-rich region separating them. Sequence analysis of this AT-rich region, using the BLASTX analysis of this sequence failed to identify any evidence of potential genes. The strong AT bias of this sequence introduces numerous stop codons strongly suggesting it is non-coding. Additional sequence flanking the left-hand side of λCY324 was extended by IPCR using *Cla*I, *Xba*I or *Hin*dIII digested then self-ligated Lp19 genomic DNA and sequence specific primers. Analysis of *ltm* cluster 3 sequence (Figure 40) identified two genes, a cytochrome P450 monooxygenase, *ltmJ,* and *ltmE,* a gene that encodes a gene fusion of two prenyl transferases, a *ltmC* type with a dimethylallyl tryptophan synthase *ltmD* type (Figure 28).

The complete *ltmJ* gene was contained on λCY234. The nucleotide sequence of *N. lolii ltm* J and deduced amino acid sequence of *ltm*Q polypeptide shown in Figure 41 and 42 respectively. Sequence analysis of *ltmJ* revealed the presence of six introns of which all are conserved with the introns, 1, 2, 3, 4, 5 and 7, from *ltmK* located in *ltm* cluster 1. LtmJ has a best BLASTP match to an *A. nidulans* AN1598 sequence (Table 8). Of the four *N. lolii* cytochrome P450 monooxygenase genes identified, LtmJ is most similar to LtmK followed by LtmP then LtmQ (Table 7).

The complete *ltmE* has significant BLASTP matches to both *P. paxilli paxC*, and to the *A. nidulans* gene, AN8514 (Table 7). The nucleotide sequence of *N. lolii ltm* E and deduced amino acid sequence of *ltm*Q polypeptide shown in Figure 43 and 44 respectively. FastA analysis shows that LtmE is 55.8 % identical to LtmC and 37.1 % identical to LtmD. The *ltmE* gene contains 3 introns (Figure 39) of which intron 1 from the *ltmC*-like domain is conserved with the placement and phase of *ltmC* intron, while intron 3, from the *ltmD*-like domain is possibly conserved with the second *ltmD* intron.

The library screen with the *ltmJ* probe isolated two identical clones λCY325 andλCY338 that were rearranged at the T7 end. These clones contained sequence

with strong similarity to class V chitin synthase gene, *chsV*. The complete gene was contained within the clones and sequenced. The gene is approximately 5.7 kb and has two introns (Figure 39) that are conserved with placement and phase to those found in other fungal *chsV* genes. The sequence of *chsV* gene is highly conserved with a significant BLASTP match to *Blumeria graminis* (accession number AAF04279) (Table 8). However, *chsV* is not part of *ltm* cluster 3.

The introns of *ltmJ* and *ltmE* from cluster 3 and the *chsV* from λCY325 and λCY338 were confirmed by RT-PCR using cDNA from endophyte infected plant material. The intron number, intron splice sites and predicted mass in kDa of each gene are summarised in Table 8.

Expression profiles of the 10 ltm genes

The expression profiles of the 10-*ltm* genes, the *chsV* from λCY325 and a polyketide synthetase adjacent to cluster 1, were characterised *in planta* and in culture. Previous data showed that the endophyte biomass *in planta* is approximately 1%. Given that expression of *ltmG*, *ltmM* and *ltmK* were highly up regulated *in planta* the other genes involved in lolitrem biosynthesis were also expected to follow a similar expression pattern. Random primed cDNA pools were made from mRNA of Lp19 infected Nui perennial ryegrass, Fl1 infected meadow fescue, and Lp19 and Fl1 grown in liquid culture. The cDNA pools from the two endophyte growth conditions, *in planta* and in culture were diluted to levels where the endophyte *tub2* sequences were amplified to similar levels thereby adjusting the levels of cDNA from endophytes grown in culture to a similar level to that of the endophyte *in planta*. A dilution series of the cDNA synthesised from mRNA of endophyte grown in culture and cDNA synthesised from mRNA of endophyte infected ryegrass diluted 1/10 were used as templates for the amplification of *tub2* with primers T1.1 and T1.2. The expression of the *tub2* gene was equivalent between cDNA from endophyte infected ryegrass

diluted 1/10 with a 1/2000 or 1/400 cDNA dilution from Lp19 or Fl1 culture conditions, respectively.

The expression pattern of each gene was subsequently compared from the cDNA of endophyte infected ryegrass, to that of cDNA from the endophyte alone. The expression of all 10 *ltm* genes have similar transcript levels indicating that these genes are highly up regulated *in planta*. No transcript was detected for any of the 10-*ltm* genes from cDNA of endophytes grown in culture. An additional dilution, 10 fold more concentrated, of cDNA synthesised from the endophyte culture was included in the experiment to unequivocally show that the expression patterns from the culture condition did not contain *ltm* transcripts. The expression of the *chsV* is similar to that of *tub2* where the gene appears to be constitutively expressed *in planta* and in culture. No evidence of *pks* expression is seen in either endophyte infected plant material or in culture.

Example 9. Functional analysis of *ltmC*

Functional characterisation of *ltmC* was determined by complementation of the *P. paxilli paxC* deletion mutant, ABC83. The ABC83 mutant is blocked early in the paxilline biosynthesis pathway and therefore unable to synthesis indole-diterpenoids (data not shown). To express *ltmC* in the *P. paxilli* background, the gene was put under the control of the *paxM* promoter in pPN1851 (Figure 45 and 29). The sequences of the Lp19 and Fl1 *ltmC* genes are identical, therefore the *ltmC* gene was amplified from Lp19 genomic DNA using the high fidelity proofreading enzyme, Platinum *Pfx* (Invitrogen), with primers lol235 and lol236. These primers, incorporate *Nco*I and *Eco*RI restriction enzyme recognition sites, respectively. The 1242 bp PCR fragment, containing the *ltmC* gene and 109-bp of 3' untranslated region, was digested with *Nco*I and *Eco*RI and directionally cloned into pPN1851, resulting in plasmid pCY34 (Figure 45). The *ltmC* gene was fused to the *paxM* promoter at the ATG translational start site using the restriction enzyme *Nco*I. The translational

fusion that results in creating an *Nco*I site in the *ltmC* gene caused a single base change where the second codon of *ltmC* has a conservative replacement of threonine in the wild-type gene, to alanine in the fused gene. A 3.5 kb *Hin*dIII fragment from λCY315 was cloned into a pUC118 vector resulting in plasmid pCY66 (Figure 45). This 3.5 kb *Hin*dIII fragment contained the complete Lp19 *ltmC* gene under the control of its native promoter. Protoplasts of ABC83 were transformed with pII99 and pJA8, containing an endogenous *paxC* fragment, or co-transformed with pCY34 and pII99, or pCY66 and pII99, and transformants selected on geneticin. Approximately 5-10 stable *P. paxilli* ABC83 transformants were colony purified and subsequently screened by TLC analysis for their ability to produce paxilline (Figure 46).

TLC analysis of the wild-type *P. paxilli* indole-diterpenoid extraction showed intense green bands that have the same Rf as paxilline, paspaline and 13-desoxypaxilline (Figure 46). The ABC83 *paxC* mutant, used for the transformations, was unable to produce any indole-diterpene (Figure 46). The ABC83 transformants containing pII99 are unable to complement the *paxC* mutation and are therefore paxilline negative (Figure 46; samples ABC283-#). The ABC83 transformants co-transformed with plasmids pII99 and pCY66 with the Lp19 *ltmC* gene under the control of the native Lp19 promoter are unable to complement the *paxC* mutation and are paxilline negative (Figure 46; samples ABC383-#). All five ABC83 transformants containing the endogenous *paxC* gene on plasmid pJA8 were able to complement the *paxC* deletion phenotype (Figure 46; samples ABC483-#). Seven of the 10 transformants containing *ltmC* under the control of the *paxM* promoter are able to produce paxilline (Figure 46; samples ABC583-#). The TLC analysis was confirmed by HPLC analysis. This data confirmed that *ltmC* is a functional orthologue of *paxC*.

Example 10. Methods for Expression of Lolitrem genes in Transgenic Plants

Knowledge of the lolitrem biosynthetic gene cluster allows for modification of the fungal genes to enable expression in transgenic plants. Fungal genes containing

introns will not be correctly spliced in plants so cDNAs for each gene need to be obtained. Those familiar with the art will know it is possible to isolate cDNAs using cDNA synthesis kits such as those described in Example 6. The cDNAs need to be cloned into a vector that contains a plant promoter and terminator sequence. Those familiar with the art know that there are many possible promoter and terminator combinations. A common example is the 35S promoter from Cauliflower Mosaic Virus (Odell et al., 1985). These modified fungal genes can then be transformed into plant species using either the gene gun or agrobacterium. Two methods are described below.

Transformation of Lolium perenne by Microprojectile bombardment of embryogenic callus

It is possible to use perennial ryegrass *L. perenne* as a model system for monocot plant species. Demonstration of biosynthesis of indole diterpenes in this species can be extrapolated to other monocot species such as wheat, rice and corn.

Materials

- florally induced tillers of *Lolium perenne*
- Na-hypochlorite (5% available chlorine)
- sterile ddH_2O100mm Petri plates containing LP5 medium*
- 100mm Petri plates containing LP3-OS medium
- 100mm Petri plates containing LP3 medium
- 100mm Petri plates containing LP3 medium + 200 mg/L Hygromycin (Hm)
- 100mm Petri plates containing MSK medium + 200 mg/L Hm
- 250 ml culture vessels containing MSO medium + 200mg/L

- Hygromycin stock solution (50 mg/ml in PDS, sterile)

Procedure

1) Harvest and surface sterilise floral tillers of *Lolium perenne* in 5% available chlorine Na-hypochlorite for 15 minutes using a Mason jar (or equivalent) under constant agitation.

2) Rinse tillers with autoclaved ddH_2O.

3) Aseptically dissect floral meristems.

4) Culture meristems on callus induction medium LP5 (16-20 explants per plate) and incubate in the dark for four to six weeks.

5) On the day of transformation transfer embryogenic callus material to high osmotic medium LP3-OS. Arrange approximately 4 cm^2 of calli in the centre of the Petri dish.

6) Incubate calli for 4-6 hours at room temperature.

7) Prepare particles and perform biolistic transformation following the protocol: "Biolistic Transformation of *Lolium perenne* with the Bio-Rad Particle Delivery System (PDS)". Plasmids are co-transformed. One plasmid (pAcH1) contains the hygromycin phosphotransferase gene conferring resistance to the antibiotic hygromycin expressed from the rice actin promoter and the second plasmid contains the genetic construct of interest for transformation. Plasmids are mixed in a one to one ratio at 1μg/μLand simultaneously coated onto the microcarriers.

8) Incubate bombarded calli on high osmotic medium LP3-OS for an additional 12-16 hours (overnight) at 25°C in the dark.

9) Transfer bombarded calli to LP3 medium and incubate for 48 hours at 25°C in the dark

10) Plate calli on selection medium (LP3 + 200 mg/l Hygromycin (Hm)). Incubate at 25°C in the dark on selection medium for two weeks.

11) Transfer all Hm-resistant callus material to regeneration medium MSK + 200 mg/l Hm and incubate for four weeks at 25°C under a 16hour photoperiod.

12) Transfer developed shoots to MS0 + 200 mg/l Hm and incubate for another two to four weeks at 25°C under 16hour photoperiod.

13) Screen by PCR Hm-resistant plants growing on MSO + 200 mg/L Hm.

Microprojectile bombardment of Lolium perenne with the Bio-Rad Particle Delivery System (PDS-1000/He)

Taken from the PDS-100/He manual. These procedures were developed by Sanford *et al.* (1992).

Materials and Solutions

- Bio-Rad Biolistic® PDS-1000/He Particle Delivery System
- Rupture disks (900 PSI)
- Macrocarriers
- Macrocarrier holders
- Microcarriers (1.0 μm)

- Stopping screens
- Autoclaved 1.5 ml eppendorf tubes
- Micropipette tips
- Vortex and microfuge
- Torque wrench tool
- Pen vac
- 70% Ethanol
- Absolute Ethanol
- 2.5 M $CaCl_2$
- 100 mM Spermidine

(A) Microcarrier preparation

For 120 bombardments using 500 µg per bombardment.

1. In a 1.5 ml microfuge tube, weigh out 60 mg of microparticles.
2. Add 1 ml of 70% ethanol, freshly prepared.
3. Vortex on a platform vortexer for 3-5 minutes.
4. Incubate for 15 minutes.
5. Pellet the microparticles by spinning for 5 seconds in a microfuge.
6. Remove the liquid and discard.
7. Repeat the following steps three times:

a. Add 1 ml of sterile water

b. Vortex for 1 minute

c. Allow the particles to settle for 1 minute

d. Pellet the microparticles by spinning for 2 seconds in a microfuge.

e. Remove the liquid and discard.

8. Add sterile 50% glycerol to bring the microparticle concentration to 60 mg/ml (assume no loss during preparation).

9. Store the microparticles at room temperature for up to 2 weeks.

(B) Coating DNA onto microcarriers

The following procedure is sufficient for six bombardments; if fewer bombardments are needed, prepare enough microcarriers for three bombardments by reducing all volumes by one half. When removing aliquots of microcarriers, it is important to vortex the tube containing the microcarriers continuously in order to maximise uniform sampling.

1. Vortex the microcarriers prepared in 50% glycerol (60 mg/ml) for 5 minutes on a platform vortexer to resuspend and disrupt agglomerated particles.

2. Remove 50 µl (3 mg) of microcarriers to a 1.5 ml microfuge tube.

3. While vortexing vigorously, add in order:

5 µl DNA (1 µg/µl)

50 µl $CaCl_2$ (2.5 M)

20 µl spermidine (0.1 M)

4. Continue vortexing for 2-3 minutes

5. Allow the microcarriers to settle for 1 minute

6. Pellet the microcarriers by spinning for 2 seconds in a microfuge

7. Remove the liquid and discard

8. Add 140 µl of 70% ethanol without disturbing the pellet

9. Remove the liquid and discard

10. Add 140 µl of 100% ethanol without disturbing the pellet

11. Remove the liquid and discard

12. Add 48 µl of 100% ethanol

13. Gently resuspend the pellet by tapping the side of the tube several times, and then by vortexing at low speed for 2-3 seconds

14. Remove six 6 µl aliquots of microcarriers and transfer them to the centre of a macrocarrier. An effort is made to remove equal amounts (500 µg) of microcarriers each time and to spread them evenly over the central 1 cm of the macrocarrier using the pipette tip. Desiccate immediately.

C) Bombardment procedure

1) Open valve of helium cylinder

2) Adjust helium regulator by turning the helium pressure regulator to 200 PSI above chosen rupture disk (e.g. if a 900 PSI rupture disk will be used, the working pressure has to be adjusted to 1100 PSI)

3) Turn on vacuum pump

4) Place 900psi rupture disk in the rupture disk-retaining cap. Screw on and tighten retaining cap.

5) Place macrocarriers in sterile macrocarrier holder

6) Place stop screen and macrocarrier holder in the launch assembly, tighten screw lid and place below rupture disk-retaining cap. Launch assembly should be set to a Gap distance of !/4 inch and macrocarrier travel distance of 11mm.

7) Place tissue sample at a target distance of 90mm.

8) Turn on main switch of PDS

9) Apply vacuum to 27 inches of Hg

10) Hold vacuum and press "fire" button until shot is performed (automatic)

11) Release "fire" button and vent chamber

12) After shooting close valve of helium cylinder and loosen pressure valve

Table 10. Compositions of the media used

Media component	LP3	LP5	LP3-OS	MSK	MS0
Macro elements (mg/l final concentration)					
KNO_3	1900	1900	1900	1900	1900
NH_4NO_3	1650	1650	1650	1650	1650
$CaCl_2 \times 2H_2O$	440	440	440	440	440
$MgSO_4 \times 2H_2O$	370	370	370	370	370
KH_2PO_4	170	170	170	170	170
KCl					
Micro elements (mg/l final concentration)					
Na_2EDTA	37.3	37.3	37.3	37.3	37.3
$FeSO_4 \times 7H_2O$	27.8	27.8	27.8	27.8	27.8
H_3BO_3	6.2	6.2	6.2	6.2	6.2
KI	0.83	0.83	0.83	0.83	0.83
$MnSO_4 \times H_2O$	16.9	16.9	16.9	16.9	16.9
$ZnSO_4 \times 7H_2O$	8.6	8.6	8.6	8.6	8.6
$CuSO_4 \times 5H_2O$	0.025	0.025	0.025	0.025	0.025
$Na_2MoO_4 \times 2H_2O$	0.25	0.25	0.25	0.25	0.25
$CoCl_2 \times 6H_2O$	0.025	0.025	0.025	0.025	0.025
Carbohydrates (g/l final concentration)					
Maltose	30	30	30	30	30
D-Mannitol			64		
Hormones (mg/l final concentration)					
2,4-D	3.0	5.0	3.0		

Kinetin				0.2	
Vitamins (mg/l final concentration) Pyridoxine HCl Thiamine HCl Nicotinic acid Myo-Inositol	 0.5 0.1 0.5 100	 0.5 0.1 0.5 100	 0.5 0.1 0.5 100	 0.5 0.1 0.5 100	
Other organics (mg/l final concentration) Glycine	 2	 2	 2	 2	 2

Culture Media

Weights and volumes required of each individual ingredient are specified in Table 10. Adjust media pH to 5.8 with KOH. The addition of a solidifying agent is required. Use agarose (for LP3, LP5 and LP3-OS) and 0.8% (w/v) Agar for MS0 and MSK prior to sterilising. Media LP3, LP5 and MSK are modified from Murashige and Skoog (1962).

Expression of chimeric genes in Corn Cells

A chimeric gene comprising a lolitrem cDNA encoding in sense orientation with respect to the promoter that is located 5' to the cDNA fragment, and a terminator 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector as described below. Amplification is then performed in a standard PCR reaction. The amplified DNA is then digested with restriction enzymes and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Sambrook, 1989). The ligated DNA may then be used to transform *E.Coli* XL1-Blue (Epicurian Coli XL-1 Blue™, Stratagent). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA sequencing Kit; US Biochemical). The resulting plasmid

construct would comprise a chimeric gene encoding in the 5' to 3' direction promoter, a cDNA encoding and the 3' region containg a terminator.

The chimeric gene described above can then be introduced into cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines. The embryos are isolated 10 to 11 days after pollination when theya re 1.0 to 1.5mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al. (1975) The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1um in diatmeter) are coated with DNA using the following technique. Ten ug of plasmid DNAs are added to 50 uL of a suspension of gold particles (60mg per mL). Calcium chloride (50 uL of a 2.5 M solution) and spermidine free base (20 uL of a 1.0M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 uL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 uL of ethanol. An aliquot (5 uL) of the DNA-coated gold particles can be placed in the centre of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments Hercules

CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The Petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardement the tissue can be transferred to N6 medium that contains a selection. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing the selection. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the selective medium. These calluses may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al., (1990)

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof.

Example 11. Use of Ltm gene sequence information to characterise endophyte strains

Identification of the ltm gene cluster allows for characterisation of endophyte strains genetically for correlation of gene information with the chemical phenotype. A number

of *Neotyphodium* and *Epichloë* strains do not produce lolitrem B. In this example we demonstrate that strains Lp1, Lp14 and AR1 that are all lolitrem B minus (data not shown) lack the *ltmE* and *ltmJ* genes of cluster 3. Southern analysis (Figure 47 A and B) showed that these strains did not hybridise to probes for the *ltmJ* or *ltmE* genes. Genes for Cluster 2 were present (Figure 3, 27, 39 and 47). This suggests that *LtmJ* and or *LtmE* are important for the biosynthesis of lolitrem B. However not all the lolitrem biosynthesis pathway is absent in these strains (Clusters 1 and 2) suggesting that intermediate compounds may be produced.

The lolitrem producers were *N. lolii* Lp19 and Lp5, and *E. festucae* Fl1. *N. lolii* Lp14 produces janthitrems, compounds structurally related to the lolitrems. The lolitrem non-producers were *N. lolii* AR1, *Neotyphodium* spp. Lp1, and *E. typhina* E8. The chemotype of *E. festucae* E189 is unknown. These isolates were screened for the presence of genes *ltmP*, *ltmJ* and *ltmE* as strains had previously been shown by Southern analysis to have differences in this region. Very little nucleotide sequence diversity is found between the asexual *N. lolii*, Lp19, and sexual *E. festucae*, Fl1, across the *ltm* genes in cluster 1, therefore standard hybridisation conditions were used.

The *ltmP* probe hybridised to seven of the eight strains screened (Fig. 47). E8 is the only strain negative for *ltmP* hybridisation. The *ltmP* probe contains a *Sst*I site and therefore hybridises to two fragments with a 9-kb hybridising band common to the seven strains that contain *ltmP*. This band in Lp19 contains the genes *ltmC*, *ltmD*, *ltmQ*, and partial sequences of *ltm25* and *ltmP* (Fig. 47). Sequence diversity is seen amongst the seven strains that contained *ltmP* as the bands of the *Eco*RI digested DNA and the second *Sst*I hybridising fragment were of varying sizes. Lp5 has two copies of *ltmP* seen clearly as two hybridising bands in the *Eco*RI digested DNA.

The exact approach is described as follows: the *ltmJ* probe hybridised to four, Lp19, Lp5, Fl1 and E189, of the eight strains screened (Fig. 47). E8, Lp1, AR1 and Lp14

are all negative for *ltmJ* hybridisation. Lp5 contains two copies of *ltmJ*, one that hybridises to a *Sst*I fragment the same size as Lp19 (~18 kb) and a second 8.5 kb fragment. The *ltmE* probe hybridises to the same four strains, Lp19, Lp5, Fl1 and E189, as that of the *ltmJ* hybridisation (Fig. 47). E8, Lp1, AR1 and Lp14 are all negative for *ltmE* hybridisation. Lp19 and Lp5 have the same sized *ltmE* hybridising band of ~20 kb. Fl1 and E189 have *ltmE* hybridising bands of 9 kb and 1.2 kb, respectively. The AT-rich region between clusters 2 and 3 is smaller in Fl1 and E189 than Lp19 based on the sizes of the hybridising bands with the *ltmP* and *ltmJ* probes (Fig. 47). Absence of *ltmJ* and *ltmE* in Lp1, AR1, Lp14 and E8 correlated with a lolitrem B negative phenotype, suggesting that these two genes are specific for lolitrem biosynthesis. A schematic diagram of the cluster 2 and 3 regions from strains used in the Southern analysis is shown in Figure 47. Attempts were made by IPCR to isolate the regions from Lp1, Lp14 and AR1 that flank the deletions but this was unsuccessful.

Similar approaches can be adopted to characterise further strains. Chemical analysis can be linked to the presence or absence of specific genes described in this specification. In addition to Southern analysis, a number of approaches could be used to detect specific genes including PCR and/or sequence analysis.

REFERENCES

Acklin W, Weibel F, Arigoni D (1977) Zur biosynthese von paspalin und verwandten metaboliten aus *Claviceps paspali*. Chimia 31:63

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403-410

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389-3402

Barbato C, Calissano M, Pickford A, Romano N, Sandmann G, Macino G (1996) Mild RIP - an alternative method for *in vivo* mutagenesisof the *albino-3* gene in *Neurospora crassa*. Mol Gen Genet 252:353-361

Belofsky GN, Gloer JB, Wicklow DT, Dowd PF (1996) Indole antiinsectan metabolites from the ascostromata of *Eupenicillium shearii*. US Patent 5,492,902

Blankenship JD, Spiering MJ, Wilkinson HH, Fannin FF, Bush LP, schardl CL (2001) Production of loline alkaloids by the grass endophyte, Neotyphodium uncinatum, in defined media. Phytochemistry 58:395-401.

Bush LP, Wilkinson HH, Schardl CL (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol 114:1-7

Carroll AM, Sweigard JA, Valent B (1994) Improved vectors for selecting resistance to hygromycin. Fungal Genet News 22

Chen AP, Kroon PA, Poulter CD (1994) Isoprenyl diphosphate synthases: protein sequence comparisons, a phylogenetic tree, and predictions of secondary structure. Protein Sci 3:600-607

(Chu et al. (1975) *Sci. Sin Peking* 18:659-668).

Cole RJ, Cox RH (1981) Handbook of toxic fungal metabolites. Academic Press, London

Creighton T. E. 'Proteins Structure and Molecular Properties.' WH Freeman and Co. 1984.

de Jesus AE, Gorst-Allman CP, Steyn PS, van Heerden FR, Vleggar R, Wessels PL, Hull WE (1983) Tremorogenic mycotoxins from *Penicillium crustosum*. Biosynthesis of Penitrem A. J. Chem. Soc. Perkin Trans. 1863-1868

Eggink G, Engel H, Vriend G, Terpstra P, Witholt B (1990) Rubredoxin reductase of *Pseudomonas oleovorans*: structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. J Mol Biol 212:135-142

Fletcher LR, Harvey IC (1981) An association of a Lolium endophyte with ryegrass staggers. N Z Vet J. 29(10):185-186

Fletcher LR (1999) "Non-toxic" endophytes in ryegrass and their effect on livestock health and production. In: Woodfield DR, Matthew C (eds) Ryegrass endophyte: an essential New Zealand symbiosis. Napier, New Zealand, pp. 133-139

Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM. (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Biotechnology 8(9):833-839.

Gallagher RT, Hawkes AD, Steyn PS, Vleggaar R (1884) Tremorgenic neurotoxing from perennial ryegrass causing ryegrass staggers disorder of livestock: structure elicidation of lolitrem B. Chem. Soc. Chemical Communications 614-616.

Gatenby WA, Munday-Finch SC, Wilkins AL, Miles CO (1999) Terpendole M, a novel indole-diterpenoid isolated from *Lolium perenne* infected with the endophytic fungus *Neotyphodium lolii*. J Agric Food Chem 47:1092-1097

Gloer JB (1995) Antiinsectan natural products from fungal sclerotia. Acc Chem Res 28:343-350

Graham-Lorence SE, Peterson JA (1996) Structural alignments of P450s and extrapolations to the unknown. Methods Enzymol 272:315-326

Higgins D, Thompson JN, Gibson TJ, Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,position-specific gap penalties and weight matrix choice. Nucl Acids Res 22:4673-4680

Huang X-H, Tomoda H, Nishida H, Masuma R, Omura S (1995) Terpendoles, novel ACAT inhibitors produced by *Albophoma yamanashiensis.* I. Production, isolation and biological properties. J Antibiotics 48:1-4

Keller NP, Hohn TM (1997) Metabolic pathway gene clusters in filamentous fungi. Fungal Genet Biol 21:17-29

Knaus H-G, McManus OB, Lee SH, Schmalhofer WA, Garcia-Calvo M, Helms LMH, Sanchez M, Giangiacomo K, Reuben JP, Smith AB, Kaczorowski GJ, Garcia ML (1994) Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated channels. Biochemistry 33:5819-5828

Krause MH, Aaronson SA (1991) Methods in Enzymology 200:546-556.

Laakso JA, Gloer JB, Wicklow DT, Dowd PF (1992) Sulpinines A-C and secopenitrem B: new antiinsectan metabolites from the sclerotia of *Aspergillus sulphurous.* J Org Chem 57:2066-2071

Latch GCM, Christensen MJ (1985) Artificial infection of grasses with endophytes. Annals of Applied Biology 107:17-24

Laws I, Mantle PG (1989) Experimental constraints in the study of the biosynthesis of indole alkaloids in fungi. J Gen Microbiol 135:2679-2692

Li C, Gloer JB, Wicklow DT, Dowd PF (2002) Thiersinines A and B: novel antiinsectan indole diterpenoids from a new fungicolous *Penicillium* species (NRRL 28147). Org Lett 4:3095-3098

Mantle PG (1987) Secondary metabolites of *Penicillium* and *Acremonium*. In: Peberdy JF (ed), *Penicillium* and *Acremonium*. Plenum Press, New York, pp. 161-243

Mantle PG, Weedon CM (1994) Biosynthesis and transformation of tremorgenic indole-diterpenoids by *Penicillium paxilli* and *Acremonium lolii*. Phytochemistry 36:1209-1217.

McLeay LM, Smith BL, Munday-Finch SC. (1999) Tremorgenic mycotoxins paxilline, penitrem and lolitrem B, the non-tremorgenic 31-epilolitrem B and electromyographic activity of the reticulum and rumen of sheep. Res Vet Sci. Apr;66(2):119-27. Erratum in: Res Vet Sci. 67(3):313.

McMillan LK, Carr RL, Young CA, Astin JW, Lowe RGT, Parker EJ, Jameson GB, Finch SC, Miles CO, McManus OB, Schmalhofer WA, Garcia ML, Kaczorowski GJ, Goetz MA, Tkacz JS, Scott B (2003) Molecular analysis of two cytochrome P450 monooxygenase genes required for paxilline biosynthesis in *Penicillium paxilli* and effects of paxilline intermediates on mammalian maxi-K ion channels. Mol Gen Genom 270:9-23

Mende K, Homann V, Tudzynski B (1997) The geranylgeranyl diphosphate synthase gene of *Gibberella fujikuroi*: isolation and expression. Mol Gen Genet 255:96-105

Munday-Finch SC, Wilkins AL, Miles CO (1996) Isolation of paspaline B, an indole-diterpenoid from *Penicillium paxilli*. Phytochemisty 41:327-332

Murashige and Skoog, 1962: Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassys with tobacco tissue cultures. Physiol Plant 15:473-497

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC. (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci U S A. 95(5):2044-2049.

Odell JT, Nagy F, Chua NH. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature. 1985 Feb 28-Mar 6;313(6005):810-2.

Ondeyka JG, Helms GL, Hensens OD, Goetz MA, Zink DL, Tsipouras A, Shoop WL, Slayton L, Dombrowski AW, Polishook JD, Ostlind DA, Tsou NN, Ball RG, Singh SB (1997) Nodulisporic acid A, a novel and potent insecticide from a *Nodulisporum* sp. Isolation, structure determination and chemical transformations. J Chem Soc Chem Commun 119:8809-8816

Parker EJ, Scott DB (2004) Indole-diterpene biosynthesis in ascomycetous fungi. In: An Z (ed), Handbook of Industrial Mycology. Marcel Dekker, New York, pp 405-426.

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444-2448

Popay AJ, Hume DE, Baltus JG, Latch GCM, Tapper BA, Lyons TB, Cooper BM, Pennell CG, Eerens JPJ, Marshall SL (1999) Field performance of perennial ryegrass (*Lolium perenne*) infected with toxin-free fungal endophytes (*Neotyphodium spp.*). In Woodfield DR, Matthew C (eds) Ryegrass endophyte: an essential New Zealand symbiosis. Napier, New Zealand, pp. 113-122.

Sanford et al., 1993:Sanford J.C., Smith, F.D., and Russell, J.A. 1993. Optimising the biolistic process for different biological applications. Methods in Enzymol. 217: 483-509

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467

Schardl CL (2001) *Epichloë festucae* and related mutualistic symbionts of grasses. Fungal Genet Biol 33:69-82

Schardl CL (1996) *Epichloë* species: fungal symbionts of grasses. Ann Rev Phytopathol 34:109-130

Scott DB (2001) *Epichloë* endophytes: symbionts of grasses. Curr Opin Microbiol 4:393-398

Scott DB, Jameson GB, Parker EJ (2003) Isoprenoids: gene clusters and biosynthetic puzzles. In: Tkacz JS, Lange L (eds), Advances in fungal biotechnology for industry, agriculture and medicine. Kluwer Academic/Plenum Publishers, New York,

Selala MI, Laekeman GM, Loenders B, Masuka A, Herman AG, Schepens P (1991) *In vitro* effects of tremorgenic mycotoxins. J Nat Products 54:207-212

Smith BL, McLeay LM, Embling PP (1997) Effect of the mycotoxins penitrem, paxilline and lolitrem B on the electromyographic activity of skeletal and gastrointestinal smooth muscle of sheep. Res Vet Sci. 62(2):111-116.

Spiering MJ, Davies E, Tapper BA, Schmid J, Lane GA (2002) Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. J Agricultural and Food Chem 50:5856-5862.

Steyn PS, Vleggaar R (1985) Tremorgenic mycotoxins. Prog Chem Organic Natural Products 48:1-80

Tomoda H, Tabata N, Yang DJ, Takayanagi H, Omura S. (1995). Terpendoles, novel ACAT inhibitors produced by Albophoma yamanashiensis. III. Production, isolation and structure elucidation of new components. J. Antibiot. 48(8):793-804.

Tudzynski B, Hölter K (1998) Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. Fungal Genet Biol 25:157-170

Vallon O (2000) New sequence motifs in flavoproteins: evidence for common ancestry and tools to predict structure. Proteins: Structure, Function and Genetics 38:95-114

Wang J, Machado C, Panaccione DG, Tsai H-F, Schardl CL (2004) The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fungal Genet Biol

Wierenga RK, Terpstra P, Hol WGJ (1986) Predictions of the occurrence of the ADP-binding bab-fold in proteins using an amino acid sequence fingerprint. J Mol Biol 187:101-107

Yao Y, Peter AB, Baur R, Sigel E (1989) The tremorgen aflatrem is a positive allosteric modulator of the gamma-aminobutyric acid receptor channel in *Xenopus* oocytes. Mol Pharmacol 35:319-323

Young CA, McMillan L, Telfer E, Scott B (2001) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. Mol Microbiol 39:754-764

Young CA, Itoh Y, Johnson R, Garthwaite I, Miles CO, Munday-Finch SC, Scott B (1998) Paxilline-negative mutants of *Penicillium paxilli* generated by heterologous and homologous plasmid integration. Curr Genet 33:368-377

Zhang S, Monahan BL, Tkacz, JS, Scott B (2004) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Aspergillus flavus*. Appl. Environ. Microbiol. 70:6875-6883.

WHAT WE CLAIM IS:

1. An isolated nucleic acid molecule having a nucleic acid sequence selected from the group consisting of:

   a) SEQ ID NOs 1, 3, 5, 17, 19, 21, 7, 9, 11, 13, 15, 52 and 54 or a combination of these sequences;

   b) SEQ ID NOs 23, 24 and 25;

   c) a functional fragment or variant of the sequences in a) or b);

   d) a complement to the sequences in a), b) or c).

2. An isolated nucleic acid molecule having at least 70% sequence homology to a nucleic acid as claimed in claim 1.

3. An isolated nucleic acid molecule having at least 80% sequence homology to a nucleic acid as claimed in claim 1.

4. An isolated nucleic acid molecule having at least 90% sequence homology to a nucleic acid as claimed in claim 1.

5. An isolated nucleic acid molecule having at least 95% sequence homology to a nucleic acid as claimed in claim 1.

6. An isolated nucleic acid molecule having at least 99% sequence homology to a nucleic acid as claimed in claim 1.

7. An isolated polypeptide having an amino acid sequence selected from the group consisting of:

   a) SEQ ID NOs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 53 and 55 or a combination of these sequences;

b) A functional fragment or variant of the sequences listed in a).

8. An isolated polypeptide molecule having at least 70% sequence homology to a polypeptide as claimed in claim 7.

9. An isolated polypeptide molecule having at least 80% sequence homology to a polypeptide as claimed in claim 7.

10. An isolated polypeptide molecule having at least 90% sequence homology to a polypeptide as claimed in claim 7.

11. An isolated polypeptide molecule having at least 95% sequence homology to a polypeptide as claimed in claim 7.

12. An isolated polypeptide molecule having at least 99% sequence homology to a polypeptide as claimed in claim 7.

13. A primer capable of specifically binding to a nucleic acid molecule selected from the group consisting of SEQ ID NO. 11 or SEQ ID NO. 12.

14. A primer having a nucleotide sequence selected from the group consisting of SEQ ID NOs 26-51.

15. A primer having a nucleotide sequence which comprises at least substantially 15-20 contiguous nucleotides of a nucleic acid molecule selected from the group consisiting of: SEQ ID NOs. 1, 3, 5, 17, 19, 21, 7, 9, 11, 13, 15, 52 and 54.

16. A probe capable of specifically binding to a nucleic acid molecule as claimed in claim 1.

17. The use of a probe capable of specifically binding to a nucleic acid molecule as claimed in claim 1 to identify at least one gene of the lolitrem gene cluster in an endophyte.

18. An isolated nucleic acid molecule which is able to stringently hybridize to a nucleic acid molecule as claimed in claim 1.

19. An isolated nucleic acid molecule as claimed in claim 18 wherein the molecule is a primer.

20. An isolated nucleic acid molecule as claimed in claim 18 wherein the molecule is a probe.

21. A method for identifying mutations in the lolitrem gene cluster of an endophyte exhibiting useful phenotypic traits, characterized by the steps of:

a) identifying at least one gene in the lolitrem gene cluster of an endophyte;

b) sequencing the gene(s) identified at a);

c) comparing the sequence at b) to SEQ ID NOs 1, 3, 5, 17, 19, 21, 7, 9, 11, 13, 15, 52 and 54 or a combination of these sequences to ascertain any differences in nucleotide sequence.

22. An endophyte in which at least one of the genes in the lolitrem gene cluster has been mutated or otherwise disrupted to manipulate the indole diterpene biosynthetic pathway.

23. The use of a nucleic acid molecule as claimed in claim 1 to produce an indole diterpene, enzyme, intermediate or other chemical compound associated with the indole diterpene biosynthetic pathway.

24. The use of a nucleic acid molecule as claimed in claim 1 to study the indole diterpene pathway.

25. A construct which includes a nucleic acid molecule as claimed in claim 1.

26. A host cell which includes a non-endogenous nucleic acid molecule as claimed in claim 1.

27. An endophyte which includes a non-endogenous nucleic acid molecule as claimed in claim 1.

28. The use of a polypeptide as claimed in claim 7 to catalyze *in vitro* or *in vivo* a reaction involved in the biosynthesis of an idole diterpene.

29. A kit for identifying the lolitrem gene cluster which includes a probe or primer capable of specifically binding to a nucleic acid sequence selected from:

(a) SEQ ID NOS. 1, 3, 5, 7,9 , 11, 13, 15, 17, 19, 21, 23, 24, 25, 52 & 54;

(b) A functional fragment or variant of the sequence in (a).

30. A kit for identifying the lolitrem gene cluster which specifically includes at least one primer pair selected from SEQ ID NOS. 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51.

31. A method of manipulating the indole diterpene biosynthetic pathway characterized by the step of altering a nucleic acid as claimed in claim 1 to produce a gene encoding a non-functional polypeptide.

32. The use of a gene produced by the method of claim 31 to manipulate the indole diterpene biosynthetic pathway.

33. An expression system which includes a non-endogenous nucleic acid molecule as claimed in claim 1.

34. The use of an expression system as claimed in claim 33 to produce indole diterpene, enzyme, intermediate or other chemical compound associated with the indole diterpene biosynthetic pathway.

35. The use of a primer as claimed in any one of claims 13-15 to specifically amplify a nucleic acid molecule.

36. A plant including a cell which includes a non-endogenous nucleic acid molecule as claimed in claim 1.

37. A plant as claimed in claim 36 wherein the plant is a grass.

38. A plant as claimed in claim 37 wherein the plant is a rye grass.

39. A plant as claimed in claim 37 or 38 wherein the cell is present as an endophyte.

40. The use of an isolated nucleic acid molecule as substantially described herein in the biosysthesis of an indole diterpene.

SEQUENCE LISTING

<110> Celentis Limited
AgResearch Limited

<120> Indole-Diterpene Biosynthesis

<130> 42616

<140> NZ 530331
<141> 2003-12-22

<160> 55

<170> PatentIn version 3.3

<210> 1
<211> 1110
<212> DNA
<213> Neotyphodium lolii

<400> 1
atgacgatgg ctgccaatga ctttccattt caatgccagg agaagaaatc atattctcag 60

ccaagtctag tctactgcaa tggtaacatt gcggagacgt atctcgaaga aaaggtattt 120

atactgctcc tttataatct cgaatgccac ttaaaattta gacaggtttt gacagcgccg 180

ttggattatt tgcgtgcctt acctagcaaa gatattcgca gtggactgac cgacgccatt 240

aatgagttcc tgcgtgtccc agaggaaaag gttcttgtca taaagcgtat aattgatctt 300

cttcacaatg catccttact gtaagttcga gattgcataa catagaccta gtagattcta 360

actaacagct ttagcattga tgatatccag gattcatcca aactgcgacg tggagtccct 420

gtagcccacc acatatttgg aatcgcacaa acaataaatt cggccaatct agcgtatttc 480

attgcccaga gagagcttga gaagcttacg aatcctcgag catttgctat atataatgag 540

gagctaatca atctgcatcg tggtcagggt atggagctcc attggagaga atcgctccat 600

tgccctaccg aagatgagta tctgcgaatg atccaaaaga agacaggcgg tctgttccga 660

ttggcaatca gactgctgca aggcgaaagc gctagcgatg acgattatgt ctcacttatt 720

gatactctcg gaaccctgtt ccagattcga gatgactatc aaaacttaca gagtgatata 780

tattctaaga acaaaggcta ctgtgaggat ttaacagagg gcaaattctc gtatccggtc 840

atccatagta ttcggtcgcg accaggagat gttcgattaa tcaatatttt gaaacagcgt 900

agtgaagatg ttatggtgaa gcaatacgcg gtgcaacata tcgaatctac aggaagcttc 960

gcattctgtc aaaataaaat tcaatctttg gtggagcaag caagagagca attggcggct 1020

ctagaaaata gcagttcatg tggaggcccc gttcgcgaca tccttgacaa gttagcaata 1080

aaaccacggg caaatataga agtagagtag 1110

<210> 2
<211> 334
<212> PRT
<213> Neotyphodium lolii

<400> 2

Met Thr Met Ala Ala Asn Asp Phe Pro Phe Gln Cys Gln Glu Lys Lys
1 5 10 15

Ser Tyr Ser Gln Pro Ser Leu Val Tyr Cys Asn Gly Asn Ile Ala Glu
20 25 30

Thr Tyr Leu Glu Glu Lys Val Leu Thr Ala Pro Leu Asp Tyr Leu Arg
35 40 45

Ala Leu Pro Ser Lys Asp Ile Arg Ser Gly Leu Thr Asp Ala Ile Asn
50 55 60

Glu Phe Leu Arg Val Pro Glu Glu Lys Val Leu Val Ile Lys Arg Ile
65 70 75 80

Ile Asp Leu Leu His Asn Ala Ser Leu Leu Ile Asp Asp Ile Gln Asp
85 90 95

Ser Ser Lys Leu Arg Arg Gly Val Pro Val Ala His His Ile Phe Gly
100 105 110

Ile Ala Gln Thr Ile Asn Ser Ala Asn Leu Ala Tyr Phe Ile Ala Gln
115 120 125

Arg Glu Leu Glu Lys Leu Thr Asn Pro Arg Ala Phe Ala Ile Tyr Asn
130 135 140

Glu Glu Leu Ile Asn Leu His Arg Gly Gln Gly Met Glu Leu His Trp
145 150 155 160

Arg Glu Ser Leu His Cys Pro Thr Glu Asp Glu Tyr Leu Arg Met Ile
165 170 175

Gln Lys Lys Thr Gly Gly Leu Phe Arg Leu Ala Ile Arg Leu Leu Gln
180 185 190

```
Gly Glu Ser Ala Ser Asp Asp Asp Tyr Val Ser Leu Ile Asp Thr Leu
        195                 200                 205

Gly Thr Leu Phe Gln Ile Arg Asp Asp Tyr Gln Asn Leu Gln Ser Asp
    210                 215                 220

Ile Tyr Ser Lys Asn Lys Gly Tyr Cys Glu Asp Leu Thr Glu Gly Lys
225                 230                 235                 240

Phe Ser Tyr Pro Val Ile His Ser Ile Arg Ser Arg Pro Gly Asp Val
                245                 250                 255

Arg Leu Ile Asn Ile Leu Lys Gln Arg Ser Glu Asp Val Met Val Lys
            260                 265                 270

Gln Tyr Ala Val Gln His Ile Glu Ser Thr Gly Ser Phe Ala Phe Cys
        275                 280                 285

Gln Asn Lys Ile Gln Ser Leu Val Glu Gln Ala Arg Glu Gln Leu Ala
    290                 295                 300

Ala Leu Glu Asn Ser Ser Ser Cys Gly Gly Pro Val Arg Asp Ile Leu
305                 310                 315                 320

Asp Lys Leu Ala Ile Lys Pro Arg Ala Asn Ile Glu Val Glu
                325                 330
```

<210> 3
<211> 1647
<212> DNA
<213> Neotyphodium lolii

<400> 3

```
atgactagcg acttcaaggt aataatcgtg ggaggatcag tggctgggct ttcactagcc     60

cactgcttag aaaaaatcgg tgtttctttc atggttctag agaagggtaa tcaaatagct    120

ccccaactcg gtgcctcaat tggcattttg ccaaatggtg gacgtattct tgatcaactg    180

ggcatcttcc atagcatcga ggatgaaatc gaacctctag aatctgctat gatgagatac    240

ccggatggtt tctctttcaa aagtcaatat ccccaagctt tgcatactag gtaataacag    300

tgaaagaaga gtggcctata agtgttcata tatcgctaac ttcgtgcggt taatagtttt    360

ggttatcccg tggctttcct tgagaggcaa aggtttcttc agatacttta tgataaactc    420
```

aagagcaaag actgcgtttt tacaaacaag cgggtagtca gtattgcaag tggccaagac 480

aaagtcacag caaagacttc agatggcgct aagtacttag cagatatcgt gatcggtgct 540

gacggggtcc acagcatcgt caggtcagag atttggaggc atttgaagga aaactctcaa 600

atatcagtat tagaggcacc gaacgcaagt aggttaacct aggattaatt gcaaagaaac 660

tttactaatg agggagccac ttaggtatta agcatgatta ttcatgcatt tacggaattt 720

ctttaaacgt tccccagatc atcctaggaa tacagttaaa ctgtttagat gacggagtgt 780

caatacactt gtttacgggt aaacaatcca aattattttg gtttgttatc atcaaaacgc 840

ctcaggctag ctttgctaaa gtagagattg acaatacaca tacagcaagg tgtatctgcg 900

aaggactgag gacgaaaaag gtttcagata ccttatgttt tgaagatgta tggtcaagat 960

gcaccatatt caagatgacg cctcttgagg aaggggtgtt taagcattgg aactatggcc 1020

gcttagcatg tattggtgat gctatccgca aggtatgtgg atgatgctat atgtccctat 1080

ttcgtgtcat cagtgggatg acaaaagaag gccactattt gccgctaata taaatgatcg 1140

tatcgctaac attaacagat ggccccaaat aatgggcaag gagcaaatat ggcgatagag 1200

gacgcttgca gtctcgcaaa catcctccag aaaaagatat cacatggttc gattcgagac 1260

caagatatca attcaatgtt tcaggaattc tctatggctc aacgggctcg cacggagagc 1320

gtctgcgcgc agtcggagtt tctagtccgc atgcatgcga atcaaggtat tggaagaaga 1380

cttcttgggc ggtaccttat tcctttcctg tatgacgcac ctgctggttt atctggattt 1440

tctataagtg gcgcaacaag aatagagttc atagacttgc ccactagatc tcttagggga 1500

gcgtggggaa agtcatggag agggtcatgg gaattcatcc tacaaagctt ggtctatttg 1560

cgacccaagt ttaggatagt ttatgccttg tatctcgttg cagctgcagc ttttatcttg 1620

tattgtctta gcagtctctt cccgtag 1647

<210> 4
<211> 472
<212> PRT
<213> Neotyphodium lolii

<400> 4

Met Thr Ser Asp Phe Lys Val Ile Ile Val Gly Gly Ser Val Ala Gly
1 5 10 15

Leu Ser Leu Ala His Cys Leu Glu Lys Ile Gly Val Ser Phe Met Val
20 25 30

Leu Glu Lys Gly Asn Gln Ile Ala Pro Gln Leu Gly Ala Ser Ile Gly
35 40 45

Ile Leu Pro Asn Gly Gly Arg Ile Leu Asp Gln Leu Gly Ile Phe His
50 55 60

Ser Ile Glu Asp Glu Ile Glu Pro Leu Glu Ser Ala Met Met Arg Tyr
65 70 75 80

Pro Asp Gly Phe Ser Phe Lys Ser Gln Tyr Pro Gln Ala Leu His Thr
85 90 95

Ser Phe Gly Tyr Pro Val Ala Phe Leu Glu Arg Gln Arg Phe Leu Gln
100 105 110

Ile Leu Tyr Asp Lys Leu Lys Ser Lys Asp Cys Val Phe Thr Asn Lys
115 120 125

Arg Val Val Ser Ile Ala Ser Gly Gln Asp Lys Val Thr Ala Lys Thr
130 135 140

Ser Asp Gly Ala Lys Tyr Leu Ala Asp Ile Val Ile Gly Ala Asp Gly
145 150 155 160

Val His Ser Ile Val Arg Ser Glu Ile Trp Arg His Leu Lys Glu Asn
165 170 175

Ser Gln Ile Ser Val Leu Glu Ala Pro Asn Ala Ser Ile Lys His Asp
180 185 190

Tyr Ser Cys Ile Tyr Gly Ile Ser Leu Asn Val Pro Gln Ile Ile Leu
195 200 205

Gly Ile Gln Leu Asn Cys Leu Asp Asp Gly Val Ser Ile His Leu Phe
210 215 220

Thr Gly Lys Gln Ser Lys Leu Phe Trp Phe Val Ile Ile Lys Thr Pro
225 230 235 240

Gln Ala Ser Phe Ala Lys Val Glu Ile Asp Asn Thr His Thr Ala Arg
245 250 255

Cys Ile Cys Glu Gly Leu Arg Thr Lys Lys Val Ser Asp Thr Leu Cys
260 265 270

Phe Glu Asp Val Trp Ser Arg Cys Thr Ile Phe Lys Met Thr Pro Leu
275 280 285

Glu Glu Gly Val Phe Lys His Trp Asn Tyr Gly Arg Leu Ala Cys Ile
290 295 300

Gly Asp Ala Ile Arg Lys Met Ala Pro Asn Asn Gly Gln Gly Ala Asn
305 310 315 320

Met Ala Ile Glu Asp Ala Cys Ser Leu Ala Asn Ile Leu Gln Lys Lys
325 330 335

Ile Ser His Gly Ser Ile Arg Asp Gln Asp Ile Asn Ser Met Phe Gln
340 345 350

Glu Phe Ser Met Ala Gln Arg Ala Arg Thr Glu Ser Val Cys Ala Gln
355 360 365

Ser Glu Phe Leu Val Arg Met His Ala Asn Gln Gly Ile Gly Arg Arg
370 375 380

Leu Leu Gly Arg Tyr Leu Ile Pro Phe Leu Tyr Asp Ala Pro Ala Gly
385 390 395 400

Leu Ser Gly Phe Ser Ile Ser Gly Ala Thr Arg Ile Glu Phe Ile Asp
405 410 415

Leu Pro Thr Arg Ser Leu Arg Gly Ala Trp Gly Lys Ser Trp Arg Gly
420 425 430

Ser Trp Glu Phe Ile Leu Gln Ser Leu Val Tyr Leu Arg Pro Lys Phe
435 440 445

Arg Ile Val Tyr Ala Leu Tyr Leu Val Ala Ala Ala Ala Phe Ile Leu
450 455 460

Tyr Cys Leu Ser Ser Leu Phe Pro
465 470

<210> 5
<211> 2063
<212> DNA
<213> Neotyphodium lolii

<400> 5
atgcaatacg gtaatttaac aactgtatta cttctgcgta atactttatt gtccttgaat 60

tcttcgtcaa tctgccatgt tcactggctg caagtgattg tggctctgct tgtcttgatc 120

gtctgcatct ttctatattg gcgaacaccc actggcatca atgctccttt cgcaggatat 180

cgttcaccat gggagccgcc gctcttggtt cagatgcgtt acgtcttcaa cgctgcctca 240

atgatacgcg aaggatatgc taaggtatgt tttatcccgc gtagaggtct tctacccgga 300

tagaccgaga agataacaac ttcggaacag tggaaagact ccttgttcca gatctcacga 360

tacgacggtg acattcttat tgtgcctcca agatatttgg atgacctcca caacaagtca 420

caagaggagt taagtgctat ttatggtttg attcgggtga ggaatgccac caaccaaaaa 480

acgcagagcc tattagcgca tggtctcaca tattcgaatt tgctagaatt ttggtggtag 540

ctatagcggc atcaccctgc ttggagaaaa cgatgttggc attcgtgcgc ttcaggtatg 600

tacacccttc caaaagtctg ttagggacct tccttactct actacagaca aaaatcaccc 660

caaatcttgc gaaattatgc gatgacataa gggatgagtt tcagtattgt ctagatacag 720

acttcccagc ctgcagaggt atgccatttc caaaatccca ttatgcagtc tctacttttt 780

ctggcactaa cgatatctaa catagattgg acatcagtgt ccgtgcatcc attgtttcta 840

aaagcagtcg aaaggataac acatcggatt tttgttggat tgccattatg tcggaatccc 900

caatgggtcc aagcgaccag caagcatgca cattacggta cgtcaattga ctaataatag 960

gcaatatacg cgctcatatg ctttgcagca acaatgatac agatagctat gagatctgtc 1020

ccaaagttca ttcagccttt actaaatttt tgccttccgt ggccatggaa gaacgcagcc 1080

tgtgttcgtg aagcaaagaa tgcccttata ttagaaatgc aacgccgacg aaatctcgag 1140

aaagttaaca gttttgatta tatcaaatcc aatgacttgc tgcaagcagt tatggaaatg 1200

tcttctccta gtcatgagga tagccagctt gatgttgtcg cccagataat gctcacgatg 1260

aacacaatcg ctggccacag tactgccgca tccggagcac atgcactgtt cgatatggtt 1320

agccactcta agtatattga attgctgcgt gaggaggctc ttcaagtctt tcgacatgtt 1380

gaactgcgtg ttacaaaaca ggctttgggg gatttgcgaa aattggacag cttcctcaga 1440

gagttagtat tgtcctaaac atcacaatct caccacattc tcacgctagc ttttcctccg 1500

tactaatgat ggtcgttgct aagatcccaa cgacataatc cgctaagctt gtgtatgttt 1560

agctaagagt ctcgaaaacc tggaaatgtt tgtcctgtgc ccgagttcta acgtctctta 1620

ctacagtagg ctttttcgg gtcgtattag accctgccgg tatcacactt caagatggca 1680

cacatgttcc ttacaacaca ctgctttgtg tcgcaccaca tgcgatatcc aatgacccgg 1740

atgtgataga agacccaacc tcgttcaacg gtctgcgata ctacgaacag cgctgtcgtg 1800

acgccagtca agagaaaaag catcaatacg ctactacgga taaatctcac ctgcattttg 1860

gctacggaac ctgggcctgt ccaggccgct tcttggcctc tgatatgtta aaagtgattc 1920

taacgatgct tctgcttcag tatgacatcc gctcccccga gagagcaaaa cggcctgtgg 1980

caggtcattt tcatgagttt ccgcttttca atattaacac accactgtta atgaaacgac 2040

gcaatgattc gctagttcta tga 2063

<210> 6
<211> 533
<212> PRT
<213> Neotyphodium lolii

<400> 6

Met Gln Tyr Gly Asn Leu Thr Thr Val Leu Leu Leu Arg Asn Thr Leu
1 5 10 15

Leu Ser Leu Asn Ser Ser Ser Ile Cys His Val His Trp Leu Gln Val
20 25 30

Ile Val Ala Leu Leu Val Leu Ile Val Cys Ile Phe Leu Tyr Trp Arg
35 40 45

Thr Pro Thr Gly Ile Asn Ala Pro Phe Ala Gly Tyr Arg Ser Pro Trp
50 55 60

Glu Pro Pro Leu Leu Val Gln Met Arg Tyr Val Phe Asn Ala Ala Ser
65 70 75 80

Met Ile Arg Glu Gly Tyr Ala Lys Trp Lys Asp Ser Leu Phe Gln Ile
85 90 95

Ser Arg Tyr Asp Gly Asp Ile Leu Ile Val Pro Pro Arg Tyr Leu Asp
100 105 110

Asp Leu His Asn Lys Ser Gln Glu Glu Leu Ser Ala Ile Tyr Gly Leu
115 120 125

Ile Arg Asn Phe Gly Gly Ser Tyr Ser Gly Ile Thr Leu Leu Gly Glu
130 135 140

Asn Asp Val Gly Ile Arg Ala Leu Gln Thr Lys Ile Thr Pro Asn Leu
145 150 155 160

Ala Lys Leu Cys Asp Asp Ile Arg Asp Glu Phe Gln Tyr Cys Leu Asp
165 170 175

Thr Asp Phe Pro Ala Cys Arg Asp Trp Thr Ser Val Ser Val His Pro
180 185 190

Leu Phe Leu Lys Ala Val Glu Arg Ile Thr His Arg Ile Phe Val Gly
195 200 205

Leu Pro Leu Cys Arg Asn Pro Gln Trp Val Gln Ala Thr Ser Lys His
210 215 220

Ala His Tyr Ala Thr Met Ile Gln Ile Ala Met Arg Ser Val Pro Lys
225 230 235 240

Phe Ile Gln Pro Leu Leu Asn Phe Cys Leu Pro Trp Pro Trp Lys Asn
245 250 255

Ala Ala Cys Val Arg Glu Ala Lys Asn Ala Leu Ile Leu Glu Met Gln
260 265 270

Arg Arg Arg Asn Leu Glu Lys Val Asn Ser Phe Asp Tyr Ile Lys Ser
275 280 285

Asn Asp Leu Leu Gln Ala Val Met Glu Met Ser Ser Pro Ser His Glu
290 295 300

Asp Ser Gln Leu Asp Val Val Ala Gln Ile Met Leu Thr Met Asn Thr
305 310 315 320

Ile Ala Gly His Ser Thr Ala Ala Ser Gly Ala His Ala Leu Phe Asp
325 330 335

Met Val Ser His Ser Lys Tyr Ile Glu Leu Leu Arg Glu Glu Ala Leu
340 345 350

Gln Val Phe Arg His Val Glu Leu Arg Val Thr Lys Gln Ala Leu Gly
355 360 365

Asp Leu Arg Lys Leu Asp Ser Phe Leu Arg Glu Ser Gln Arg His Asn
370 375 380

Pro Leu Ser Leu Leu Gly Phe Phe Arg Val Val Leu Asp Pro Ala Gly
385 390 395 400

Ile Thr Leu Gln Asp Gly Thr His Val Pro Tyr Asn Thr Leu Leu Cys
405 410 415

Val Ala Pro His Ala Ile Ser Asn Asp Pro Asp Val Ile Glu Asp Pro
420 425 430

Thr Ser Phe Asn Gly Leu Arg Tyr Tyr Glu Gln Arg Cys Arg Asp Ala
435 440 445

Ser Gln Glu Lys Lys His Gln Tyr Ala Thr Thr Asp Lys Ser His Leu
450 455 460

His Phe Gly Tyr Gly Thr Trp Ala Cys Pro Gly Arg Phe Leu Ala Ser
465 470 475 480

Asp Met Leu Lys Val Ile Leu Thr Met Leu Leu Leu Gln Tyr Asp Ile
485 490 495

Arg Ser Pro Glu Arg Ala Lys Arg Pro Val Ala Gly His Phe His Glu
500 505 510

Phe Pro Leu Phe Asn Ile Asn Thr Pro Leu Leu Met Lys Arg Arg Asn
515 520 525

Asp Ser Leu Val Leu
530

<210> 7
<211> 1115
<212> DNA
<213> Neotyphodium lolii

<400> 7
atgacatctg gagcatggct cgtggctcgc cctgcggcca tcgaaattgc ggccctcttg 60

tttgcattta cgctcgggta tctagtaaag tacacaatca attaccaatc tgtcgtttct 120

caagccattg atcattatgg ctatggctat gaacgtacct ctcacgaagg tattggcggc 180

agcaatggca agattcctga ctgtccatac tcttatgtga ttagtctcta tgggcataat 240

catttctctc ccctcgtgga ttttcttcat ccaacattga aacataaata tcccaagaaa 300

cattctttga tcctggatat catggatgcg gtccatcttt gtctaattat ggttgacgat 360

atttgcgacc acagccctaa gcggaaaaat cacactacgg ctcacttgct atacggatca 420

tgcgaaactg ccaatcgagc atacttcgtt ctcacaaagg tcattaatag agcaatgaaa 480

gaacaacctg tccttggaat tgaacttctg agagcactag aactgatact cgagggacaa 540

gacatgtctt tggtttggcg aagagacggt ttgcgatctt tcgaatccta tggtgaagaa 600

agcctgttga cgtacaaaaa tatggctctg ctgaagacag gcacactttt tgtgctcctt 660

gggaggcttt tgaaccaagg aggtcatcaa tcagacgatc tgttaggccg atttgggtat 720

gtaaattttt ttttttttcg ctcgtttcat aattccgcgg caaggtcgct taactaattc 780

aatggaatgg tagctggtac gcacaattgc aaaatgattg caagaacata tactcagaag 840

agtacgcttt taacaaaggc actgttgcag aagacctacg caacagagaa ttgtcctttc 900

ctgttgtggt tgctcttaat gacaaacata ctgagccgca gataaggaag gcgtttcaga 960

gccaaaatca aggcgacatt aaacgggcac tccaagcgtt agagtcacct agtgttaaaa 1020

acacgtgtct caaaacgctc caggaggcag gtcagggtct agagaacttg gtggccgtct 1080

ggggacgaaa agaacaaatg cactttacaa aatga 1115

<210> 8
<211> 345
<212> PRT
<213> Neotyphodium lolii

<400> 8

Met Thr Ser Gly Ala Trp Leu Val Ala Arg Pro Ala Ala Ile Glu Ile
1 5 10 15

Ala Ala Leu Leu Phe Ala Phe Thr Leu Gly Tyr Leu Val Lys Tyr Thr
20 25 30

Ile Asn Tyr Gln Ser Val Val Ser Gln Ala Ile Asp His Tyr Gly Tyr
35 40 45

Gly Tyr Glu Arg Thr Ser His Glu Gly Ile Gly Gly Ser Asn Gly Lys

50 55 60

Ile Pro Asp Cys Pro Tyr Ser Tyr Val Ile Ser Leu Tyr Gly His Asn
65 70 75 80

His Phe Ser Pro Leu Val Asp Phe Leu His Pro Thr Leu Lys His Lys
85 90 95

Tyr Pro Lys Lys His Ser Leu Ile Leu Asp Ile Met Asp Ala Val His
100 105 110

Leu Cys Leu Ile Met Val Asp Asp Ile Cys Asp His Ser Pro Lys Arg
115 120 125

Lys Asn His Thr Thr Ala His Leu Leu Tyr Gly Ser Cys Glu Thr Ala
130 135 140

Asn Arg Ala Tyr Phe Val Leu Thr Lys Val Ile Asn Arg Ala Met Lys
145 150 155 160

Glu Gln Pro Val Leu Gly Ile Glu Leu Leu Arg Ala Leu Glu Leu Ile
165 170 175

Leu Glu Gly Gln Asp Met Ser Leu Val Trp Arg Arg Asp Gly Leu Arg
180 185 190

Ser Phe Glu Ser Tyr Gly Glu Glu Ser Leu Leu Thr Tyr Lys Asn Met
195 200 205

Ala Leu Leu Lys Thr Gly Thr Leu Phe Val Leu Leu Gly Arg Leu Leu
210 215 220

Asn Gln Gly Gly His Gln Ser Asp Asp Leu Leu Gly Arg Phe Gly Trp
225 230 235 240

Tyr Ala Gln Leu Gln Asn Asp Cys Lys Asn Ile Tyr Ser Glu Glu Tyr
245 250 255

Ala Phe Asn Lys Gly Thr Val Ala Glu Asp Leu Arg Asn Arg Glu Leu
260 265 270

Ser Phe Pro Val Val Val Ala Leu Asn Asp Lys His Thr Glu Pro Gln
275 280 285

Ile Arg Lys Ala Phe Gln Ser Gln Asn Gln Gly Asp Ile Lys Arg Ala
290 295 300

Leu Gln Ala Leu Glu Ser Pro Ser Val Lys Asn Thr Cys Leu Lys Thr
305 310 315 320

Leu Gln Glu Ala Gly Gln Gly Leu Glu Asn Leu Val Ala Val Trp Gly
325 330 335

Arg Lys Glu Gln Met His Phe Thr Lys
340 345

<210> 9
<211> 1829
<212> DNA
<213> Neotyphodium lolii

<400> 9
atgttaatgt tgcacgctgt cccagtgggt atctgtttac tactgtggta cgttgtttac 60

ggtaccaaac ggaaagaatg tataccaacc attcgacgtt ggcctcgatt actcccccaa 120

ttcctcgatc ggctgagtta taatgaccat gccgcccgcc tagtcaaaca tggctatgag 180

aaggtgttcg gtcccaagtc tgtgagcatc cgtgatgata gtactaaccg cctaggttac 240

agcacaaaaa tcaaccgttt aggctactta agatggacat ggatctgatt gtcattcctt 300

tacaatacgc gctggaatta cgggcggtta cgagcgacaa attagaccct ttaacagcca 360

gctttgatga caatgctggt aaagttacga ggatattatt agggagcgaa cttcacacac 420

gtgccataca gcagcgtttg actccaaagc ttcgtaagtg taatctaaca gaatacgttt 480

gcactatgct aactgaatcc agcacaaact cttccagtgc tattggatga gctcaatcat 540

gcctttgggc aagtcttacc tgccggcaac gacggtatgt gttttcattc tttcaaacat 600

tccacttgtc tagtgttgtc taatattagt ggaggttcca atgcttggat ttctgtcaat 660

ccatacgaat tggttctcaa tctagctacc cgtgctacag cgaggctatt cgttggagac 720

ctgatttgtc gaaacgaaat ttttctcgag actactgctt cttttagtcg caacacgttt 780

gatacgatat ccacctcccg tagttttggc aatttgttca cacattattt cgcacggtgg 840

atttccacag cgaaagaagc tcacgggcaa ttacaataca ttcaaaacct ccttggttca 900

gaagtccaga gaaggaaact taactctgag gaaaagcacg acgacttttt gcagtggtgt 960

acagagttag cagtcaccga ggatgaagca cggccagaag cacttgcgca tcgtacgcta 1020

ggaatattga gcatggctgt cattcataca acagctatgg cgttaactca catacttttt 1080

gacatgatct cggacgacag cttgaaggag agcctccgaa gagaacagca aaacgtgctc 1140

aagcatggtt ggacggaaat cacgcaacag actatgcttg atatgaaaca attggatagc 1200

ctgatgagag agtcacaacg aatcaatcca gtgggcgagt gtaaggacta ttctaagcca 1260

gtcttttgtc aaacttggaa ctaactggcg attgaagtca cttttagacg cattgtccga 1320

gaacgaatta cattgtccga tggctaccag ctacagccgg gacagcagat tgcgattcca 1380

gcgaagtgta tcaatacgga cagtacgaaa ttatccgacg ctcacttgtt tcaacctttt 1440

cgatggttga aacaatctgg cactgccaca acatcatttt ctaacagcag cgccttgaat 1500

ctgcacttcg gatttgggag atatgcctgt ccgggacgct tcatagcttc cgtatgtgat 1560

gtagattttc atctttttt tttccatatc aatctccctt caagctcatg tgacgcacat 1620

tcgaccttct tgactaaccc ttgagtttgt gctcatagta tatgattaaa gcaatcatga 1680

gtcggattct gctcgagtat gattttaagc tagatagtga gtttccgtcg cggcgccctc 1740

ctaacattgt tcatggggat aagatcctcc ccaatcggaa tgccgttgtt cttttgcgcc 1800

gcttggagaa gacagttacc gtatgttga 1829

<210> 10
<211> 498
<212> PRT
<213> Neotyphodium lolii

<400> 10

Met Leu Met Leu His Ala Val Pro Val Gly Ile Cys Leu Leu Leu Trp
1 5 10 15

Tyr Val Val Tyr Gly Thr Lys Arg Lys Glu Cys Ile Pro Thr Ile Arg
20 25 30

Arg Trp Pro Arg Leu Leu Pro Gln Phe Leu Asp Arg Leu Ser Tyr Asn
35 40 45

Asp His Ala Ala Arg Leu Val Lys His Gly Tyr Glu Lys His Lys Asn
50 55 60

Gln Pro Phe Arg Leu Leu Lys Met Asp Met Asp Leu Ile Val Ile Pro
65 70 75 80

Leu Gln Tyr Ala Leu Glu Leu Arg Ala Val Thr Ser Asp Lys Leu Asp
85 90 95

Pro Leu Thr Ala Ser Phe Asp Asp Asn Ala Gly Lys Val Thr Arg Ile
100 105 110

Leu Leu Gly Ser Glu Leu His Thr Arg Ala Ile Gln Gln Arg Leu Thr
115 120 125

Pro Lys Leu Pro Gln Thr Leu Pro Val Leu Leu Asp Glu Leu Asn His
130 135 140

Ala Phe Gly Gln Val Leu Pro Ala Gly Asn Asp Gly Ser Asn Ala Trp
145 150 155 160

Ile Ser Val Asn Pro Tyr Glu Leu Val Leu Asn Leu Ala Thr Arg Ala
165 170 175

Thr Ala Arg Leu Phe Val Gly Asp Leu Ile Cys Arg Asn Glu Ile Phe
180 185 190

Leu Glu Thr Thr Ala Ser Phe Ser Arg Asn Thr Phe Asp Thr Ile Ser
195 200 205

Thr Ser Arg Ser Phe Gly Asn Leu Phe Thr His Tyr Phe Ala Arg Trp
210 215 220

Ile Ser Thr Ala Lys Glu Ala His Gly Gln Leu Gln Tyr Ile Gln Asn
225 230 235 240

Leu Leu Gly Ser Glu Val Gln Arg Arg Lys Leu Asn Ser Glu Glu Lys
245 250 255

His Asp Asp Phe Leu Gln Trp Cys Thr Glu Leu Ala Val Thr Glu Asp
260 265 270

Glu Ala Arg Pro Glu Ala Leu Ala His Arg Thr Leu Gly Ile Leu Ser
275 280 285

Met Ala Val Ile His Thr Thr Ala Met Ala Leu Thr His Ile Leu Phe
290 295 300

Asp Met Ile Ser Asp Asp Ser Leu Lys Glu Ser Leu Arg Arg Glu Gln

305 310 315 320

Gln Asn Val Leu Lys His Gly Trp Thr Glu Ile Thr Gln Gln Thr Met
325 330 335

Leu Asp Met Lys Gln Leu Asp Ser Leu Met Arg Glu Ser Gln Arg Ile
340 345 350

Asn Pro Val Gly Glu Phe Thr Phe Arg Arg Ile Val Arg Glu Arg Ile
355 360 365

Thr Leu Ser Asp Gly Tyr Gln Leu Gln Pro Gly Gln Gln Ile Ala Ile
370 375 380

Pro Ala Lys Cys Ile Asn Thr Asp Ser Thr Lys Leu Ser Asp Ala His
385 390 395 400

Leu Phe Gln Pro Phe Arg Trp Leu Lys Gln Ser Gly Thr Ala Thr Thr
405 410 415

Ser Phe Ser Asn Ser Ser Ala Leu Asn Leu His Phe Gly Phe Gly Arg
420 425 430

Tyr Ala Cys Pro Gly Arg Phe Ile Ala Ser Tyr Met Ile Lys Ala Ile
435 440 445

Met Ser Arg Ile Leu Leu Glu Tyr Asp Phe Lys Leu Asp Ser Glu Phe
450 455 460

Pro Ser Arg Arg Pro Pro Asn Ile Val His Gly Asp Lys Ile Leu Pro
465 470 475 480

Asn Arg Asn Ala Val Val Leu Leu Arg Arg Leu Glu Lys Thr Val Thr
485 490 495

Val Cys

<210> 11
<211> 1945
<212> DNA
<213> Neotyphodium lolii

<400> 11

atggcatttg caagtctttt gcaccatatc tggaaccatg cagtggattg cgctgagcag 60
ctgacttggt ggcagaccat tgtgagcttc atcattttct gcatcatgtg ctcttggcta 120
cctgggaatg gggaaatgcg cgctccgttt gttggttatc gctggccatt cgagcctact 180
ttctgggtcc gaatgcgctt catctttcag agtttaggca tgatgaccga aggatactca 240
aaggtgagct cccgtccggg tggagaaaga cagctagacg aatgactgac gccaaacgct 300
tgacagttca aggattccat gttcaagatc acgaccaacg atgccgactg gcttgtcctc 360
tcccaacgct acttggatga cttgcagtct ctgccagccg agagattgag ccatacagac 420
gctctagtga cggtgagggc gcatactagt cgctagtccc tacgacagtg gtgtgctaat 480
cgagttgtgt ctcatttaga tgtgggggag cagccacagc ccttttgctc tgctcaacaa 540
gagtgatctt agctctcgag ctcttcgtgt aaggaccaat ccctccttgt tatgcagaac 600
ggatctgact tgaaaaggac gtggttgcgc cgaattatgc caaggacctt gatagcctcg 660
tagacgaact ccgctattcg cttgagcacg atatagacat acaggatggt atgtatgcgc 720
ctattttcca actaattttg aggtcgtcat gttggctgac tgggtcgatg cgcttagact 780
ggaaaccgat tgatgccctt gaactttctt cgaagttggt gttgcggata tcgcagcgaa 840
tcttgatcgg ctggcccatg agtcgcgatc aagagctcct tgaatgcgca caaggctacg 900
cagacgctgg taagaggacg agctgttacg tatgaccctt ttcttcggta aaaactaacg 960
ggggtttcag ctaccgtcgt ccagtttgcc ctgaaactac ttcctcgcca gattcggccg 1020
cttgtctatc ctctgctccc acaagcatgg gctactaaat cgtggatcag gcgctgtgac 1080
aagatactgg caaaggaaat gcaacgtcga caagttttgg agaagtcgga tcccgtgtac 1140
gagaaaccaa aggacttgct gcagggcatg gtggacctgg agccgtcccg gcctgttgac 1200
aaacttggac atgattttct cgtccaagcc ttgatttcca gaatggctcc agttgttacc 1260
atggcccaaa cccttgttga tcttgccctc catcctgagg atatcgagga gctgcgtgat 1320
gaggttctgc aagtcatagg accagacggg gcgggattag gaaacctacg acaatcattt 1380
accaaacttg acaagatgga cagcgtcttg agggaatctg ccaggttcac ccctctatct 1440
atgagtaagt gccatttctg tcctccagaa tagcttgctg gcatgactaa tctgtggtat 1500
agtgacaatg caccgccggg ttcaggacgc caagggcatc acgctccatg acggtgtgca 1560
tcttccacga ggcacgcatg tggcattccc agcgtaccac attggcagag atcccaagtt 1620
ggtgtcaggt gcagatatct atgacgggct gcgctggtac aggaaggacc tcggcgaggc 1680
ccaagaaaac gaagctccca agcatcgatt tgtcaccccc gacagcaact acttgacctt 1740

tgggtccggt aaatacgtct gccccggccg atttatagcg gaacacatgt tgaagctgat 1800

gatgaccgcc gtgctcctgc gctacgagtt caagtggcct ccgggagtcc ctgtgcccga 1860

acaacagtat cggcatgtct ttgcttatcc aagcaaaacc acactgttga ttaaacgacg 1920

caaagatggc gatcagattc tttaa 1945

<210> 12
<211> 525
<212> PRT
<213> Neotyphodium lolii

<400> 12

Met Ala Phe Ala Ser Leu Leu His His Ile Trp Asn His Ala Val Asp
1 5 10 15

Cys Ala Glu Gln Leu Thr Trp Trp Gln Thr Ile Val Ser Phe Ile Ile
20 25 30

Phe Cys Ile Met Cys Ser Trp Leu Pro Gly Asn Gly Glu Met Arg Ala
35 40 45

Pro Phe Val Gly Tyr Arg Trp Pro Phe Glu Pro Thr Phe Trp Val Arg
50 55 60

Met Arg Phe Ile Phe Gln Ser Leu Gly Met Met Thr Glu Gly Tyr Ser
65 70 75 80

Lys Phe Lys Asp Ser Met Phe Lys Ile Thr Thr Asn Asp Ala Asp Trp
85 90 95

Leu Val Leu Ser Gln Arg Tyr Leu Asp Asp Leu Gln Ser Leu Pro Ala
100 105 110

Glu Arg Leu Ser His Thr Asp Ala Leu Val Thr Met Trp Gly Ser Ser
115 120 125

His Ser Pro Phe Ala Leu Leu Asn Lys Ser Asp Leu Ser Ser Arg Ala
130 135 140

Leu Arg Asp Val Val Ala Pro Asn Tyr Ala Lys Asp Leu Asp Ser Leu
145 150 155 160

Val Asp Glu Leu Arg Tyr Ser Leu Glu His Asp Ile Asp Ile Gln Asp
165 170 175

Asp Trp Lys Pro Ile Asp Ala Leu Glu Leu Ser Ser Lys Leu Val Leu
180 185 190

Arg Ile Ser Gln Arg Ile Leu Ile Gly Trp Pro Met Ser Arg Asp Gln
195 200 205

Glu Leu Leu Glu Cys Ala Gln Gly Tyr Ala Asp Ala Ala Thr Val Val
210 215 220

Gln Phe Ala Leu Lys Leu Leu Pro Arg Gln Ile Arg Pro Leu Val Tyr
225 230 235 240

Pro Leu Leu Pro Gln Ala Trp Ala Thr Lys Ser Trp Ile Arg Arg Cys
245 250 255

Asp Lys Ile Leu Ala Lys Glu Met Gln Arg Arg Gln Val Leu Glu Lys
260 265 270

Ser Asp Pro Val Tyr Glu Lys Pro Lys Asp Leu Leu Gln Gly Met Val
275 280 285

Asp Leu Glu Pro Ser Arg Pro Val Asp Lys Leu Gly His Asp Phe Leu
290 295 300

Val Gln Ala Leu Ile Ser Arg Met Ala Pro Val Val Thr Met Ala Gln
305 310 315 320

Thr Leu Val Asp Leu Ala Leu His Pro Glu Asp Ile Glu Glu Leu Arg
325 330 335

Asp Glu Val Leu Gln Val Ile Gly Pro Asp Gly Ala Gly Leu Gly Asn
340 345 350

Leu Arg Gln Ser Phe Thr Lys Leu Asp Lys Met Asp Ser Val Leu Arg
355 360 365

Glu Ser Ala Arg Phe Thr Pro Leu Ser Met Met Thr Met His Arg Arg
370 375 380

Val Gln Asp Ala Lys Gly Ile Thr Leu His Asp Gly Val His Leu Pro

385 390 395 400

Arg Gly Thr His Val Ala Phe Pro Ala Tyr His Ile Gly Arg Asp Pro
405 410 415

Lys Leu Val Ser Gly Ala Asp Ile Tyr Asp Gly Leu Arg Trp Tyr Arg
420 425 430

Lys Asp Leu Gly Glu Ala Gln Glu Asn Glu Ala Pro Lys His Arg Phe
435 440 445

Val Thr Pro Asp Ser Asn Tyr Leu Thr Phe Gly Ser Gly Lys Tyr Val
450 455 460

Cys Pro Gly Arg Phe Ile Ala Glu His Met Leu Lys Leu Met Met Thr
465 470 475 480

Ala Val Leu Leu Arg Tyr Glu Phe Lys Trp Pro Pro Gly Val Pro Val
485 490 495

Pro Glu Gln Gln Tyr Arg His Val Phe Ala Tyr Pro Ser Lys Thr Thr
500 505 510

Leu Leu Ile Lys Arg Arg Lys Asp Gly Asp Gln Ile Leu
515 520 525

<210> 13
<211> 2014
<212> DNA
<213> Neotyphodium lolii

<400> 13
atgaagatgt taacagagca ttttgacttt cctaaactta acttcgccac cattgtaatt 60

tcaggcgcca ccattattgg tataatattc cttcgatatc ttaattaccc tacaaaggtt 120

tgaatctgtt tcccttgcta tgcacttttc ttggatgctc accaaaattt ttcaaggtga 180

atgtacctgt tgttggcatt ggagttcgat atacaaaatg gctagcggct attataaacg 240

tgcgtcatgc tcgacaatct atccgcgagg gctatgcaaa ggtttgtgtt aaaaacgaat 300

aaaagcgctt cgtaaacaaa gagaactaat actagtttct agtatggcga tttcgcgttt 360

cagataccta ctatgactcg aatggaggta ttcatttgtg atagacagat gacaagggag 420

tatcagaatg ttgacgacta tcatttgtcg ttccgagctg tcatgaccga ggtaagtaac 480

tagaccatgt taactgtagg aaaagaagaa aaagctaaac cgccgtacag gagtttcaat 540

tcaaatggct acttccagga caggcacacg aagcccggat tatccctaac tcagtgattg 600

ctaaggcctt gagctggcag agaacaaggg cgaataaacc cagcgatcca ttcttcgaat 660

ctttctccgc cgaattcatg caggggtttc aggaagagat gcgacgacta atccaatatc 720

aaaattcgtc agttatgtca aaccgctccg gtgctgtcct ggatccagcg catggttggc 780

atgctgtgcc ttgttttccc ttggctctga aggtaattgg gcgccttact acatacgtct 840

tgttcggcaa acctttgtgc caagatgcga cattcctaaa catgtgctgt caatttggcg 900

atgtgattcc cagggatgcg atcatactac gttcatggcc agcattggca aggccgtaag 960

caagtgccta gacataaacc cgtcagggtt taaactcgca ttaacattca tatagtctta 1020

ttgtaaagat cttgagtgct ccaagggtta tgggaaagtt gcgaaacatt ttgattgttg 1080

agataaagag caggagagaa tcccacgaaa cgaacccaat gagtgtatgg ctgtctcgca 1140

cacccctct agcattacac attaacgtat atctaggata tcttggattt cacaatggcc 1200

tgggttgacc gtcatcctaa cgctagcttt gacgatcagc acattgccga gatgatgatt 1260

aacactattt tcgcagctct tcatacgtcg agtcaggtat attttttttct gtatgaaaag 1320

tccagagctt aaagctaact ggctcatagc tggtggtgca taccatcttt gagcttgcct 1380

cacgtcctga atatagcgat gcgcttctgg aagagataga tgcatgcttt gaaaagcatg 1440

gaaagggcac taaagcagct ctagactcaa tgttcaaggt ggatagtttc atcaaagaaa 1500

cgcagaggtt taaccctctt gacgcatgta taaattccct gtctccgatt ccatcattgc 1560

gatttgacta acgccaccgt cagccgctct tgcaagactg gctctcaaag actttacttt 1620

ttccaatggc ctaaacatcc caaagggcag tgtgattttc acgccgaatt cgcctatctt 1680

tgaggacgag agatattaca aggatccgaa agtttttgat ggatttcggt ttgctaggat 1740

gcgtaatgac ccaaaattag gtctattctg cgacctaaca gcaacgaatg aacaaagcat 1800

gcattttggg actggacgtc acgcctgtcc tggtagattt atggtttctg atgaggtcaa 1860

gttagctgtg attcatatct taagtaattt cgatttttgt attgagaatt ttggaccacg 1920

gccagcaaat cagccatttg gtaaatttct tctacctgat atgagtgcaa aaatctggct 1980

aagggagaaa agagctaggg agaagaatct gtga 2014

<210> 14
<211> 537
<212> PRT
<213> Neotyphodium lolii

<400> 14

Met Lys Met Leu Thr Glu His Phe Asp Phe Pro Lys Leu Asn Phe Ala
1 5 10 15

Thr Ile Val Ile Ser Gly Ala Thr Ile Ile Gly Ile Ile Phe Leu Arg
20 25 30

Tyr Leu Asn Tyr Pro Thr Lys Val Asn Val Pro Val Val Gly Ile Gly
35 40 45

Val Arg Tyr Thr Lys Trp Leu Ala Ala Ile Ile Asn Val Arg His Ala
50 55 60

Arg Gln Ser Ile Arg Glu Gly Tyr Ala Lys Tyr Gly Asp Phe Ala Phe
65 70 75 80

Gln Ile Pro Thr Met Thr Arg Met Glu Val Phe Ile Cys Asp Arg Gln
85 90 95

Met Thr Arg Glu Tyr Gln Asn Val Asp Asp Tyr His Leu Ser Phe Arg
100 105 110

Ala Val Met Thr Glu Glu Phe Gln Phe Lys Trp Leu Leu Pro Gly Gln
115 120 125

Ala His Glu Ala Arg Ile Ile Pro Asn Ser Val Ile Ala Lys Ala Leu
130 135 140

Ser Trp Gln Arg Thr Arg Ala Asn Lys Pro Ser Asp Pro Phe Phe Glu
145 150 155 160

Ser Phe Ser Ala Glu Phe Met Gln Gly Phe Gln Glu Glu Met Arg Arg
165 170 175

Leu Ile Gln Tyr Gln Asn Ser Ser Val Met Ser Asn Arg Ser Gly Ala
180 185 190

Val Leu Asp Pro Ala His Gly Trp His Ala Val Pro Cys Phe Pro Leu
195 200 205

Ala Leu Lys Val Ile Gly Arg Leu Thr Thr Tyr Val Leu Phe Gly Lys
210 215 220

Pro Leu Cys Gln Asp Ala Thr Phe Leu Asn Met Cys Cys Gln Phe Gly
225 230 235 240

Asp Val Ile Pro Arg Asp Ala Ile Ile Leu Arg Ser Trp Pro Ala Leu
245 250 255

Ala Arg Pro Leu Ile Val Lys Ile Leu Ser Ala Pro Arg Val Met Gly
260 265 270

Lys Leu Arg Asn Ile Leu Ile Val Glu Ile Lys Ser Arg Arg Glu Ser
275 280 285

His Glu Thr Asn Pro Met Ser Asp Ile Leu Asp Phe Thr Met Ala Trp
290 295 300

Val Asp Arg His Pro Asn Ala Ser Phe Asp Asp Gln His Ile Ala Glu
305 310 315 320

Met Met Ile Asn Thr Ile Phe Ala Ala Leu His Thr Ser Ser Gln Leu
325 330 335

Val Val His Thr Ile Phe Glu Leu Ala Ser Arg Pro Glu Tyr Ser Asp
340 345 350

Ala Leu Leu Glu Glu Ile Asp Ala Cys Phe Glu Lys His Gly Lys Gly
355 360 365

Thr Lys Ala Ala Leu Asp Ser Met Phe Lys Val Asp Ser Phe Ile Lys
370 375 380

Glu Thr Gln Arg Phe Asn Pro Leu Asp Ala Ser Ala Leu Ala Arg Leu
385 390 395 400

Ala Leu Lys Asp Phe Thr Phe Ser Asn Gly Leu Asn Ile Pro Lys Gly
405 410 415

Ser Val Ile Phe Thr Pro Asn Ser Pro Ile Phe Glu Asp Glu Arg Tyr
420 425 430

Tyr Lys Asp Pro Lys Val Phe Asp Gly Phe Arg Phe Ala Arg Met Arg
435 440 445

Asn Asp Pro Lys Leu Gly Leu Phe Cys Asp Leu Thr Ala Thr Asn Glu
450 455 460

Gln Ser Met His Phe Gly Thr Gly Arg His Ala Cys Pro Gly Arg Phe
465 470 475 480

Met Val Ser Asp Glu Val Lys Leu Ala Val Ile His Ile Leu Ser Asn
485 490 495

Phe Asp Phe Cys Ile Glu Asn Phe Gly Pro Arg Pro Ala Asn Gln Pro
500 505 510

Phe Gly Lys Phe Leu Leu Pro Asp Met Ser Ala Lys Ile Trp Leu Arg
515 520 525

Glu Lys Arg Ala Arg Glu Lys Asn Leu
530 535

<210> 15
<211> 1496
<212> DNA
<213> Neotyphodium lolii

<400> 15
atgattgcga aaaatattga actcaatggc ttggatccgg caaccagggc attggacatt 60

ctatactgga aaaatcactg catcaaacag ctagaatctc tcctatgcgc cacagattca 120

tactgcactg cagacaaggc cgctcaacta cgcattttgt cagagttggt gctccccaat 180

cttggccctc ggccgtccaa tgccactggg ccatcctatc ttacacgaag tggttcccca 240

ataatgttaa gtctaaatac aacatcatca aaaaactgcg tcagatattg ctgggagatt 300

ctaggggcga ctggcgcaag taatgatgat cctttggcag tccaagttgc taaggatgta 360

gtggcttctc tgtctgctac ttttcgcctt tcaacaaaat ggagcgaaac tctactgtcc 420

aattttgcag taacaccaga ccaagctcga caagttatta acatgctacc cgagtggatt 480

caaggcttcg tacctgaggg aatggagtgc gattttccaa agagaatccc gttcgccatg 540

acatcattcg acctaaatgg ctccaatgta gctatgaagc tctacgttaa tccaagggta 600

aaggagattt taactggtac tccctcatca gacttggtct gggagttcct ccgaaattta 660

acaccagaaa tgaaaccacg agcggtcgac ttgcttgaga ggtaagaatg gctttgaact 720

ttcgcccacc ttgtcagccc catacgctaa gcgctaactc cccacacatt aacaggttta 780

ttaccgataa ttcaggcccg tctgctattg agcttgtagg tattgactgc gttgacgacg 840

ctcacctatc aaatgcaagg gtcaagcttt acgttcatac catgagcagc tcatttaaca 900

ccgtaaagaa ttatgttact cttgggggtg caatctggga tgaacaaacc caaaagggct 960

taggaatact acaaagtatt tggcacctat tgcttcagga gccagagggt atttctgaca 1020

atggattcga caagcctgtg aacgactctt ccatgttatg ccaaaagcta tattttagtt 1080

tcgagctacg cccaggtaca gacttccctc aggtgaagac ctatgtgcca acttggaact 1140

atcttcgaac cgacggggaa actatccaga actatgaggc gatcttccga gcttgtgacc 1200

atccttgggg tgaagatagg acgtacggca aaatttttca agatgcattg taagttatcc 1260

cttcagatta gcgctaaaag gagtttgaga tactcctcaa tgcaagctat taggttgtga 1320

aattgccact actaattgga gctttttata gcggacctgc aaccgagagt cggaaaaaac 1380

ccattcactg cgacgcatct tttctgttta ccgaagaaac tggtgtctac cagacgctgt 1440

atttcagtcc tccgattgag ggggaaacag aagtccagtc aaatctcgtt gcttga 1496

<210> 16
<211> 439
<212> PRT
<213> Neotyphodium lolii

<400> 16

Met Ile Ala Lys Asn Ile Glu Leu Asn Gly Leu Asp Pro Ala Thr Arg
1 5 10 15

Ala Leu Asp Ile Leu Tyr Trp Lys Asn His Cys Ile Lys Gln Leu Glu
20 25 30

Ser Leu Leu Cys Ala Thr Asp Ser Tyr Cys Thr Ala Asp Lys Ala Ala
35 40 45

Gln Leu Arg Ile Leu Ser Glu Leu Val Leu Pro Asn Leu Gly Pro Arg
50 55 60

Pro Ser Asn Ala Thr Gly Pro Ser Tyr Leu Thr Arg Ser Gly Ser Pro
65 70 75 80

Ile Met Leu Ser Leu Asn Thr Thr Ser Ser Lys Asn Cys Val Arg Tyr
85 90 95

Cys Trp Glu Ile Leu Gly Ala Thr Gly Ala Ser Asn Asp Asp Pro Leu

100 105 110

Ala Val Gln Val Ala Lys Asp Val Val Ala Ser Leu Ser Ala Thr Phe
115 120 125

Arg Leu Ser Thr Lys Trp Ser Glu Thr Leu Leu Ser Asn Phe Ala Val
130 135 140

Thr Pro Asp Gln Ala Arg Gln Val Ile Asn Met Leu Pro Glu Trp Ile
145 150 155 160

Gln Gly Phe Val Pro Glu Gly Met Glu Cys Asp Phe Pro Lys Arg Ile
165 170 175

Pro Phe Ala Met Thr Ser Phe Asp Leu Asn Gly Ser Asn Val Ala Met
180 185 190

Lys Leu Tyr Val Asn Pro Arg Val Lys Glu Ile Leu Thr Gly Thr Pro
195 200 205

Ser Ser Asp Leu Val Trp Glu Phe Leu Arg Asn Leu Thr Pro Glu Met
210 215 220

Lys Pro Arg Ala Val Asp Leu Leu Glu Arg Phe Ile Thr Asp Asn Ser
225 230 235 240

Gly Pro Ser Ala Ile Glu Leu Val Gly Ile Asp Cys Val Asp Asp Ala
245 250 255

His Leu Ser Asn Ala Arg Val Lys Leu Tyr Val His Thr Met Ser Ser
260 265 270

Ser Phe Asn Thr Val Lys Asn Tyr Val Thr Leu Gly Gly Ala Ile Trp
275 280 285

Asp Glu Gln Thr Gln Lys Gly Leu Gly Ile Leu Gln Ser Ile Trp His
290 295 300

Leu Leu Leu Gln Glu Pro Glu Gly Ile Ser Asp Asn Gly Phe Asp Lys
305 310 315 320

Pro Val Asn Asp Ser Ser Met Leu Cys Gln Lys Leu Tyr Phe Ser Phe
325 330 335

Glu Leu Arg Pro Gly Thr Asp Phe Pro Gln Val Lys Thr Tyr Val Pro
340 345 350

Thr Trp Asn Tyr Leu Arg Thr Asp Gly Glu Thr Ile Gln Asn Tyr Glu
355 360 365

Ala Ile Phe Arg Ala Cys Asp His Pro Trp Gly Glu Asp Arg Thr Tyr
370 375 380

Gly Lys Ile Phe Gln Asp Ala Phe Gly Pro Ala Thr Glu Ser Arg Lys
385 390 395 400

Lys Pro Ile His Cys Asp Ala Ser Phe Leu Phe Thr Glu Glu Thr Gly
405 410 415

Val Tyr Gln Thr Leu Tyr Phe Ser Pro Pro Ile Glu Gly Glu Thr Glu
420 425 430

Val Gln Ser Asn Leu Val Ala
435

<210> 17
<211> 1110
<212> DNA
<213> Epichloe festucae

<400> 17
atgacgatgg ctgccaatga ctttccattt caatgccagg agaagaaatc atattctcag 60

ccaagtctag tctactgcaa tggtaacatt gcggagacgt atctcgaaga aaaggtattt 120

atactgctcc tttataatct cgaatgccac ttaaaattta gacaggtttt gacagcgccg 180

ttggattatt tgcgtgcctt acctagcaaa gatattcgca gtggactgac cgacgccatt 240

aatgagttcc tgcgtgtccc agaggaaaag gttcttgtca taaagcgtat aattgatctt 300

cttcacaatg catccttact gtaagttcga gattgcataa catagaccta gtagattcta 360

actaacagct ttagcattga tgatatccag gattcatcta aactgcgacg tggagtccct 420

gtagcccacc acatatttgg aatcgcacaa acaataaatt cggccaatct agcgtatttc 480

attgcccaga gagagcttga gaagcttacg aatcctcgag catttgctat atataatgag 540

gagctaatca atctgcatcg tggtcagggt atggagctcc attggagaga atcgctccat 600

tgccctaccg aagatgagta tctgcgaatg atccaaaaga agacaggcgg tctgttccga 660

ttggcaatca gactgctgca aggcgaaagc gctagcgatg acgattatgt ctcacttatt 720

gatactctcg gaaccctgtt ccagattcga gatgactatc aaaacttaca gagtgatata 780

tattctaaga acaaaggcta ctgtgaggat ttaacagagg gcaaattctc gtatccggtc 840

atccatagta ttcggtcgcg accaggagat gttcgattaa tcaatatttt gaaacagcgt 900

agtgaagatg ttatggtgaa gcaatacgcg gtgcaacata tcgaatctac aggaagcttc 960

gcattctgtc aaaataaaat tcaatctttg gtggagcaag caagagagca attggcggct 1020

ctagaaaata gcagttcatg tggaggcccc gttcgcgaca tccttgacaa gttagcaata 1080

aaaccacggg caaatatagа agtagagtag 1110

<210> 18
<211> 334
<212> PRT
<213> Epichloe festucae

<400> 18

Met Thr Met Ala Ala Asn Asp Phe Pro Phe Gln Cys Gln Glu Lys Lys
1 5 10 15

Ser Tyr Ser Gln Pro Ser Leu Val Tyr Cys Asn Gly Asn Ile Ala Glu
20 25 30

Thr Tyr Leu Glu Glu Lys Val Leu Thr Ala Pro Leu Asp Tyr Leu Arg
35 40 45

Ala Leu Pro Ser Lys Asp Ile Arg Ser Gly Leu Thr Asp Ala Ile Asn
50 55 60

Glu Phe Leu Arg Val Pro Glu Glu Lys Val Leu Val Ile Lys Arg Ile
65 70 75 80

Ile Asp Leu Leu His Asn Ala Ser Leu Leu Ile Asp Asp Ile Gln Asp
85 90 95

Ser Ser Lys Leu Arg Arg Gly Val Pro Val Ala His His Ile Phe Gly
100 105 110

Ile Ala Gln Thr Ile Asn Ser Ala Asn Leu Ala Tyr Phe Ile Ala Gln
115 120 125

Arg Glu Leu Glu Lys Leu Thr Asn Pro Arg Ala Phe Ala Ile Tyr Asn
130 135 140

Glu Glu Leu Ile Asn Leu His Arg Gly Gln Gly Met Glu Leu His Trp
145 150 155 160

Arg Glu Ser Leu His Cys Pro Thr Glu Asp Glu Tyr Leu Arg Met Ile
165 170 175

Gln Lys Lys Thr Gly Gly Leu Phe Arg Leu Ala Ile Arg Leu Leu Gln
180 185 190

Gly Glu Ser Ala Ser Asp Asp Asp Tyr Val Ser Leu Ile Asp Thr Leu
195 200 205

Gly Thr Leu Phe Gln Ile Arg Asp Asp Tyr Gln Asn Leu Gln Ser Asp
210 215 220

Ile Tyr Ser Lys Asn Lys Gly Tyr Cys Glu Asp Leu Thr Glu Gly Lys
225 230 235 240

Phe Ser Tyr Pro Val Ile His Ser Ile Arg Ser Arg Pro Gly Asp Val
245 250 255

Arg Leu Ile Asn Ile Leu Lys Gln Arg Ser Glu Asp Val Met Val Lys
260 265 270

Gln Tyr Ala Val Gln His Ile Glu Ser Thr Gly Ser Phe Ala Phe Cys
275 280 285

Gln Asn Lys Ile Gln Ser Leu Val Glu Gln Ala Arg Glu Gln Leu Ala
290 295 300

Ala Leu Glu Asn Ser Ser Ser Cys Gly Gly Pro Val Arg Asp Ile Leu
305 310 315 320

Asp Lys Leu Ala Ile Lys Pro Arg Ala Asn Ile Glu Val Glu
325 330

<210> 19
<211> 1647
<212> DNA
<213> Epichloe festucae

<400> 19
atgactagcg acttcaaggt aataatcgtg ggaggatcag tggctgggct ttcactagcc 60
cactgcttag aaaaaatcgg tgtttctttc gtggttctag agaagggtaa tcaaatagct 120
ccccaactcg gtgcctcaat tggcattttg ccaaatggtg gacgtattct tgatcaactg 180
ggcatcttcc atagcatcga ggatgaaatc gaacctctag aatctgctat gatgagatac 240
ccggatggct tctctttcaa aagtcaatat ccccaagctt tgcatactag gtaataacag 300
tgaaagaaga gtggcctata agtgttcata tatcgctaac ttcgtgcggt taatagtttt 360
ggttatcccg tggctttcct tgagaggcaa aggtttcttc agatacttta tgataaactc 420
aagagcaaag actgcgtttt tacaaacaag cgggtagtca gtattgcaag tggccaagac 480
aaagtcacag caaagacttc agatggcgct aagtacttag cagatatcgt gatcggtgct 540
gacggggtcc acagcatcgt caggtcagag atttggaggc atttgaagga aaactctcaa 600
atatcagtat tagaggcacc gaacgcaagt aggttaacct aggattaatt gcaaagaaac 660
tttactaatg agggagccac ttaggtatta agcatgatta ttcatgcatt tacggaattt 720
ctttaaacgt tccccagatc atcctaggaa tacagttaaa ctgtttagat gacggagtgt 780
caatacactt gtttacgggt aaacaatcca aattattttg gtttgttatc atcaaaacgc 840
ctcaggctag ctttgctaaa gtagagattg acaatacaca tacagcaagg tgtatctgcg 900
aaggactgag gacgaaaaag gtttcagata ccttatgttt tgaagatgta tggtcaagat 960
gcaccatatt caagatgacg cctcttgagg aaggggtgtt taagcattgg aactatggcc 1020
gcttagcatg tattggtgat gctatccgca aggtatgtgg atgatgctat atgtccctat 1080
ttcgtgtcat cagtgggatg acaaaagaag gccactattt gccgctaata taaatgatcg 1140
tatcgctaac attaacagat ggccccaaat aatgggcaag gagcaaatat ggcgatagag 1200
gacgcttgca gtctcgcaaa catcctccag aaaaagatat cacatggttc gattcgagac 1260
caagatatca attcaatgtt tcaggaattc tctatggctc aacgggctcg cacggagagc 1320
gtctgcgcgc agtcggagtt tctagtccgc atgcatgcga atcaaggtat tggaagaaga 1380
cttcttgggc ggtaccttat tcctttcctg tatgacgcac ctgctggttt atctggattt 1440
tctataagtg gcgcaacaag aatagagttc atagacttgc ccactagatc tcttagggga 1500
gcgtggggaa agtcatggag agggtcatgg gaattcatcc tacaaagctt ggtctatttg 1560
cgacccaagt ttaggatagt ttatgccttg tatctcgttg cagctgcagc ttttatcttg 1620
tattgtctta gcagtctctt cccgtag 1647

<210> 20
<211> 472
<212> PRT
<213> Epichloe festucae

<400> 20

Met Thr Ser Asp Phe Lys Val Ile Ile Val Gly Gly Ser Val Ala Gly
1 5 10 15

Leu Ser Leu Ala His Cys Leu Glu Lys Ile Gly Val Ser Phe Val Val
20 25 30

Leu Glu Lys Gly Asn Gln Ile Ala Pro Gln Leu Gly Ala Ser Ile Gly
35 40 45

Ile Leu Pro Asn Gly Gly Arg Ile Leu Asp Gln Leu Gly Ile Phe His
50 55 60

Ser Ile Glu Asp Glu Ile Glu Pro Leu Glu Ser Ala Met Met Arg Tyr
65 70 75 80

Pro Asp Gly Phe Ser Phe Lys Ser Gln Tyr Pro Gln Ala Leu His Thr
85 90 95

Ser Phe Gly Tyr Pro Val Ala Phe Leu Glu Arg Gln Arg Phe Leu Gln
100 105 110

Ile Leu Tyr Asp Lys Leu Lys Ser Lys Asp Cys Val Phe Thr Asn Lys
115 120 125

Arg Val Val Ser Ile Ala Ser Gly Gln Asp Lys Val Thr Ala Lys Thr
130 135 140

Ser Asp Gly Ala Lys Tyr Leu Ala Asp Ile Val Ile Gly Ala Asp Gly
145 150 155 160

Val His Ser Ile Val Arg Ser Glu Ile Trp Arg His Leu Lys Glu Asn
165 170 175

Ser Gln Ile Ser Val Leu Glu Ala Pro Asn Ala Ser Ile Lys His Asp
180 185 190

Tyr Ser Cys Ile Tyr Gly Ile Ser Leu Asn Val Pro Gln Ile Ile Leu

195 200 205

Gly Ile Gln Leu Asn Cys Leu Asp Asp Gly Val Ser Ile His Leu Phe
210 215 220

Thr Gly Lys Gln Ser Lys Leu Phe Trp Phe Val Ile Ile Lys Thr Pro
225 230 235 240

Gln Ala Ser Phe Ala Lys Val Glu Ile Asp Asn Thr His Thr Ala Arg
245 250 255

Cys Ile Cys Glu Gly Leu Arg Thr Lys Lys Val Ser Asp Thr Leu Cys
260 265 270

Phe Glu Asp Val Trp Ser Arg Cys Thr Ile Phe Lys Met Thr Pro Leu
275 280 285

Glu Glu Gly Val Phe Lys His Trp Asn Tyr Gly Arg Leu Ala Cys Ile
290 295 300

Gly Asp Ala Ile Arg Lys Met Ala Pro Asn Asn Gly Gln Gly Ala Asn
305 310 315 320

Met Ala Ile Glu Asp Ala Cys Ser Leu Ala Asn Ile Leu Gln Lys Lys
325 330 335

Ile Ser His Gly Ser Ile Arg Asp Gln Asp Ile Asn Ser Met Phe Gln
340 345 350

Glu Phe Ser Met Ala Gln Arg Ala Arg Thr Glu Ser Val Cys Ala Gln
355 360 365

Ser Glu Phe Leu Val Arg Met His Ala Asn Gln Gly Ile Gly Arg Arg
370 375 380

Leu Leu Gly Arg Tyr Leu Ile Pro Phe Leu Tyr Asp Ala Pro Ala Gly
385 390 395 400

Leu Ser Gly Phe Ser Ile Ser Gly Ala Thr Arg Ile Glu Phe Ile Asp
405 410 415

Leu Pro Thr Arg Ser Leu Arg Gly Ala Trp Gly Lys Ser Trp Arg Gly
420 425 430

Ser Trp Glu Phe Ile Leu Gln Ser Leu Val Tyr Leu Arg Pro Lys Phe
435 440 445

Arg Ile Val Tyr Ala Leu Tyr Leu Val Ala Ala Ala Ala Phe Ile Leu
450 455 460

Tyr Cys Leu Ser Ser Leu Phe Pro
465 470

<210> 21
<211> 2063
<212> DNA
<213> Epichloe festucae

<400> 21
atgcaatacg gtaatttaac aactgtatta cttctgcgta atactttatt gtccttgaat 60

tcttcgtcaa tctgccatgt tcactggctg caagtgattg tggctctgct tgtcttgatc 120

gtctgcatct ttctatattg gcgaacaccc actggcatca atgctccttt cgcaggatat 180

cgttcaccat gggagccgcc gctcttggtt cagatgcgtt acgtcttcaa cgctgcctca 240

atgatacgcg aaggatatgc taaggtatgt tttatcccgc gtagaggtct tctacccgga 300

tagaccgaga agataacaac ttcggaacag tggaaagact ccttgttcca gatctcacga 360

tacgacggtg acattcttat tgtgcctcca agatatttgg atgacctcca caacaagtca 420

caagaggagt taagtgctat ttatggtttg attcgggtga ggaatgccac caaccaaaaa 480

acgcagagcc tattagcgca tggtctcaca tattcgaatt tgctagaatt ttggtggtag 540

ctatagcggc atcaccctgc ttggagaaaa cgatgttggc attcgtgcgc ttcaggtatg 600

tacacccttc caaaagtctg ttagggacct tccttactct actacagaca aaaatcaccc 660

caaatcttgc gaaattatgc gatgacataa gggatgagtt tcagtattgt ctagatacag 720

acttcccagc ctgcagaggt atgccatttc caaaatccca ttatgcagtc tctacttttt 780

ctggcactaa cgatatctaa catagattgg acatcagtgt ccgtgcatcc attgtttcta 840

aaagcagtcg aaaggataac acatcggatt tttgttggat tgccattatg tcggaatccc 900

caatgggtcc aagcgaccag caagcatgca cattacggta cgtcaattga ctaataatag 960

gcaatatacg cgctcatatg ctttgcagca acaatgatac agatagctat gagatctgtc 1020

ccaaagttca ttcagccttt actaaatttt tgccttccgt ggccatggaa gaacgcagcc 1080

tgtgttcgtg aagcaaagaa tgcccttata ttagaaatgc aacgccgacg aaatctcgag 1140

aaagttaaca gttttgatta tatcaaatcc aatgacttgc tgcaagcagt tatggaaatg 1200

tcttctccta gtcatgagga tagccagctt gatgttgtcg cccagataat gctcacgatg 1260

aacacaatcg ctggccacag tactgccgca tccggagcac atgcactgtt cgatatggtt 1320

agccactcta agtatattga attgctgcgt gaggaggctc ttcaagtctt tcgacatgtt 1380

gaactgcgtg ttacaaaaca ggctttgggg gatttgcgaa aattggacag cttcctcaga 1440

gagttagtat tgtcctaaac atcacaatct caccacattc tcacgctagc ttttcctccg 1500

tactaatgat ggtcgttgct aagatcccaa cgacataatc cgctaagctt gtgtatgttt 1560

agctaagagt ctcgaaaacc tggaaatgtt tgtcctgtgc ccgagttcta acgtctctta 1620

ctacagtagg ctttttcgg gtcgtattag accctgccgg tatcacactt caagatggca 1680

cacatgttcc ttacaacaca ctgctttgtg tcgcaccaca tgcgatatcc aatgacccgg 1740

atgtgataga agacccaacc tcgttcaacg gtctgcgata ctacgaacag cgctgtcgtg 1800

acgccagtca agagaaaaag catcaatacg ctactacgga taaatctcac ctgcattttg 1860

gctacggaac ctgggcctgt ccaggccgct tcttggcctc tgatatgtta aaagtgattc 1920

taacgatgct tctgcttcag tatgacatcc gctcccccga gagagcaaaa cggcctgtgg 1980

caggtcattt tcatgagttt ccgcttttca atattaacac accactgtta atgaaacgac 2040

gcaatgattc gctagttcta tga 2063

<210> 22
<211> 533
<212> PRT
<213> Epichloe festucae

<400> 22

Met Gln Tyr Gly Asn Leu Thr Thr Val Leu Leu Leu Arg Asn Thr Leu
1 5 10 15

Leu Ser Leu Asn Ser Ser Ser Ile Cys His Val His Trp Leu Gln Val
20 25 30

Ile Val Ala Leu Leu Val Leu Ile Val Cys Ile Phe Leu Tyr Trp Arg
35 40 45

Thr Pro Thr Gly Ile Asn Ala Pro Phe Ala Gly Tyr Arg Ser Pro Trp
50 55 60

Glu Pro Pro Leu Leu Val Gln Met Arg Tyr Val Phe Asn Ala Ala Ser
65 70 75 80

Met Ile Arg Glu Gly Tyr Ala Lys Trp Lys Asp Ser Leu Phe Gln Ile
85 90 95

Ser Arg Tyr Asp Gly Asp Ile Leu Ile Val Pro Pro Arg Tyr Leu Asp
100 105 110

Asp Leu His Asn Lys Ser Gln Glu Glu Leu Ser Ala Ile Tyr Gly Leu
115 120 125

Ile Arg Asn Phe Gly Gly Ser Tyr Ser Gly Ile Thr Leu Leu Gly Glu
130 135 140

Asn Asp Val Gly Ile Arg Ala Leu Gln Thr Lys Ile Thr Pro Asn Leu
145 150 155 160

Ala Lys Leu Cys Asp Asp Ile Arg Asp Glu Phe Gln Tyr Cys Leu Asp
165 170 175

Thr Asp Phe Pro Ala Cys Arg Asp Trp Thr Ser Val Ser Val His Pro
180 185 190

Leu Phe Leu Lys Ala Val Glu Arg Ile Thr His Arg Ile Phe Val Gly
195 200 205

Leu Pro Leu Cys Arg Asn Pro Gln Trp Val Gln Ala Thr Ser Lys His
210 215 220

Ala His Tyr Ala Thr Met Ile Gln Ile Ala Met Arg Ser Val Pro Lys
225 230 235 240

Phe Ile Gln Pro Leu Leu Asn Phe Cys Leu Pro Trp Pro Trp Lys Asn
245 250 255

Ala Ala Cys Val Arg Glu Ala Lys Asn Ala Leu Ile Leu Glu Met Gln
260 265 270

Arg Arg Arg Asn Leu Glu Lys Val Asn Ser Phe Asp Tyr Ile Lys Ser
275 280 285

Asn Asp Leu Leu Gln Ala Val Met Glu Met Ser Ser Pro Ser His Glu

290 295 300

Asp Ser Gln Leu Asp Val Val Ala Gln Ile Met Leu Thr Met Asn Thr
305 310 315 320

Ile Ala Gly His Ser Thr Ala Ala Ser Gly Ala His Ala Leu Phe Asp
325 330 335

Met Val Ser His Ser Lys Tyr Ile Glu Leu Leu Arg Glu Glu Ala Leu
340 345 350

Gln Val Phe Arg His Val Glu Leu Arg Val Thr Lys Gln Ala Leu Gly
355 360 365

Asp Leu Arg Lys Leu Asp Ser Phe Leu Arg Glu Ser Gln Arg His Asn
370 375 380

Pro Leu Ser Leu Leu Gly Phe Phe Arg Val Val Leu Asp Pro Ala Gly
385 390 395 400

Ile Thr Leu Gln Asp Gly Thr His Val Pro Tyr Asn Thr Leu Leu Cys
405 410 415

Val Ala Pro His Ala Ile Ser Asn Asp Pro Asp Val Ile Glu Asp Pro
420 425 430

Thr Ser Phe Asn Gly Leu Arg Tyr Tyr Glu Gln Arg Cys Arg Asp Ala
435 440 445

Ser Gln Glu Lys Lys His Gln Tyr Ala Thr Thr Asp Lys Ser His Leu
450 455 460

His Phe Gly Tyr Gly Thr Trp Ala Cys Pro Gly Arg Phe Leu Ala Ser
465 470 475 480

Asp Met Leu Lys Val Ile Leu Thr Met Leu Leu Leu Gln Tyr Asp Ile
485 490 495

Arg Ser Pro Glu Arg Ala Lys Arg Pro Val Ala Gly His Phe His Glu
500 505 510

Phe Pro Leu Phe Asn Ile Asn Thr Pro Leu Leu Met Lys Arg Arg Asn
515 520 525

Asp Ser Leu Val Leu
530

<210> 23
<211> 11400
<212> DNA
<213> Neotyphodium lolii

<400> 23
aatggactag aaagtacatt tgttatacag tgctatctcc ttaggctcag tctaccttgt 60
gggtcagtgc aggccccaca ggccccctgc cacaaggtta gtaaccgcgc aagcacgcga 120
aagtgtagcg tagtaaatta tataggaaaa attagcagta tattaattat tagcctatct 180
atatataagt aaatatacct ttaattcact tctatttaat tggatataga ccctagttaa 240
cgtgacttca caaggtgaac taagtccaag aagatagagg taattgcagt gagatccaca 300
ggtcttgtca ggggacggca atgtatgcat atatcgtgaa atcaatgcta gcggcattga 360
atcaatgact tctgtagcta gcgataatag cagcgataga agcctctaga atctatatag 420
acagtattaa gtaaactctc cacctgtatc cacagctaac ttacatacac ctagccctgt 480
cttgagtgct tttagaagac tatgctaact tagatcacac cctaagtgcc aatgtctccc 540
aattagccgc gaagagagaa cttatcgcaa ggaagtgata aggctataac atccaacagg 600
ttacttaaag acaacaggct aggaatcaat tatagtagca atcaaaacta gatcctgtat 660
tctataacaa gaagttaaat ccccccctaga ctatctgtct atctttagtt atactttggt 720
tttgctttgt tgtcttatgc ctacattcct aaaagatctt tatgacgatg gctgccaatg 780
actttccatt tcaatgccag gagaagaaat catattctca gccaagtcta gtctactgca 840
atggtaacat tgcggagacg tatctcgaag aaaaggtatt tatactgctc ctttataatc 900
tcgaatgcca cttaaaattt agacaggttt tgacagcgcc gttggattat ttgcgtgcct 960
tacctagcaa agatattcgc agtggactga ccgacgccat taatgagttc ctgcgtgtcc 1020
cagaggaaaa ggttcttgtc ataaagcgta taattgatct tcttcacaat gcatccttac 1080
tgtaagttcg agattgcata acatagacct agtagattct aactaacagc tttagcattg 1140
atgatatcca ggattcatcc aaactgcgac gtggagtccc tgtagcccac cacatatttg 1200
gaatcgcaca aacaataaat tcggccaatc tagcgtattt cattgcccag agagagcttg 1260
agaagcttac gaatcctcga gcatttgcta tatataatga ggagctaatc aatctgcatc 1320
gtggtcaggg tatggagctc cattggagag aatcgctcca ttgccctacc gaagatgagt 1380

atctgcgaat gatccaaaag aagacaggcg gtctgttccg attggcaatc agactgctgc 1440
aaggcgaaag cgctagcgat gacgattatg tctcacttat tgatactctc ggaaccctgt 1500
tccagattcg agatgactat caaaacttac agagtgatat atattctaag aacaaaggct 1560
actgtgagga tttaacagag ggcaaattct cgtatccggt catccatagt attcggtcgc 1620
gaccaggaga tgttcgatta atcaatattt tgaaacagcg tagtgaagat gttatggtga 1680
agcaatacgc ggtgcaacat atcgaatcta caggaagctt cgcattctgt caaaataaaa 1740
ttcaatcttt ggtggagcaa gcaagagagc aattggcggc tctagaaaat agcagttcat 1800
gtggaggccc cgttcgcgac atccttgaca agttagcaat aaaaccacgg gcaaatatag 1860
aagtagagta gttgacatta agaacattgc gataaaagac acttttacta tactcgacta 1920
gttttaaaac tatgtgtgag attaagacgt cttcaggtac tcaaagtgtg gaagtatgtc 1980
acgcagaaaa gagctaacat tgctctcagc ttcctcacta tttagtttca ccaagagcat 2040
ccttcataga gacatttgcg gctgtgattt tcgtttacgt catgttgtta aacattgttg 2100
tatggtatct ttgcttagga gtagacatcc attttctctc actctactct tagagatcgt 2160
caagtgtcac atacatttct gagaactagg actttgcata gaatatgcat cggttaggtg 2220
tttgcgtaga gagtacgtgt gtctgaggtt agccattgcg cttcgtttgc ggtttacaat 2280
ggggcaaggc ttaaagcttt ttaaagccac ggtgaccact actgcaggtg cattcttttt 2340
ttagtcgtaa aacactaagt tttttttact agttataata gacttttctt tccttcttcc 2400
cttctcgtag ataaacccaa ttgaagaatt aatataaagt gtattcttaa tcctagcctt 2460
atccctaaat atatatatat atattgtata ctctagctag ctctatgtag ggctagttct 2520
agtactgcct ctagttagtt aaaagggaaa acccttaaat aagaagaaaa atccctttat 2580
attttgtcag gcgaaaacaa ccacccgaaa acgacggatt tgacgatgac actaacaaca 2640
aagctaacga atttgacgat attagcaatt gaacctagat atcgggatct aggtctgcga 2700
ggtttccgat ccacgcctag gattcaagct agggggtagg gtctttttct aataataggt 2760
tattttatta attaaacaat ccaagcctaa ggcaacgaag ggagagtaaa gtttctattt 2820
aaagggaggg aatctagggg ttttatctag ctaggaggtc acatgactag ggatccgatg 2880
tggccgaatt gatctgacaa gccaatagat ctgacgaagc caaggtctag gggcccgagg 2940
tcttgtgaga gaggtctcga gaggtcacaa tgctagccac acaatatcta tcaatatatg 3000
aatatattat attatatgat ttaccctaga tagcaattta tgccattaac cagtactcct 3060

gccgtgatgt tgctttgtag taggaaaacc atactaggtt gctaattatc tagataacta 3120
gataactagt tagttgccta gttagaactc gtatctcaaa tccctgttac gtatctctct 3180
acccgcagtc ctttttagat cttgttattg agtctcgtag aagtagcaca tccgcgctac 3240
ctgcagctgg accagctatg agactgacaa aaaacatcct taccataact cgtaagctca 3300
agtgtttatt ttctgcttca agtgcttgag aaaatagccc cacggtcaag aaaaatccac 3360
ttgatgtacc agtcatctca ttaatctgtc tgagtctagc atgtcgtgca gcgatctcgg 3420
aacacggaaa ctgcgagcaa tcgggtacac caaggaggct attccctata tgaaagggag 3480
cagtggcgtc tctgtgaagg agagtcgcca cgatcgctac cataaaaatg ccaatgtggc 3540
ttataccagt gcaccagaaa atagtcctta ggaaagcctt ctcttgcctc ctcggccacg 3600
ctgttactaa tttctcggca cgatattgat ttaggatcca cagtgaaaag acgggaaagg 3660
cagtggaaag tccaactgtg taagagagat agcctagtgc ggccaaactt cttcaaaaag 3720
taagcatagt cagtgagtca gagttaacag ggaatcacat actcaaactt gcggaggaat 3780
gcgccatgcg gtacggtctc atgcagaatt atcaaaatga gcccaaccag ctgagcaatg 3840
taaagcatta ggtgaagcca aaaccaaggc ccattatccc aaatggactg catcgacgca 3900
acagcgcgaa acccgaacca tggtgatgtg gttccatagc ttaatgtagc atccgaagaa 3960
tcaatgaact gtaatgggca gggaaagtca atgatcggat atccttcccg tgacttccat 4020
attacgccgg ctaaacaaaa gaaaccctgc agagagataa agatccaatc acttcgcgac 4080
atagggaaaa atagaggaaa actgataata actttaggtc cagtttcatg caatattggg 4140
aaaggccaga agcataatcc gtacaatcgt catgatatcg tcaaagcgag actaagctgt 4200
ttctttatag gggctgagaa atcttggcaa taggaaaccg gaagaatgcc gagtgcgact 4260
gacgcaaaga attggcttga gcacccgacc ccctctccat ccctaacccg tgtcgtcatt 4320
atctttcggc aatagatatg gcgtttcatt tcactgtaac atacagatta ctccgtattt 4380
atgtaataat acaccctatt acatgtaata ttacacgtag ggaggggggtg attaggaagc 4440
gtgcggatga tacgtagaac tactatataa ttaactactc cgtatagata gctagtatta 4500
gttattgtaa aggtaggggt caatatagat gattaaaagc gttcaattta gtcaattaga 4560
ggtgcagaca gcacctgagt tttgtaccta aaaggtacat agtgcgctat agtaatgact 4620
agtttacgga ggtacttcta atacattgta tccactcgtt gtcttagaga gagttttatc 4680
ctagtcaatg cgcgctgcct catacatcct aggctttaag ggagctctcc ctgacagtta 4740
ttgcagctac cttagctaca ttcaggggtg ctatttacgc ataagggtgt gcttaataaa 4800

cacacccctg tcaataccca agccacaata aagacagttt ttgtctttgt gcagattcgt 4860
gaatcctact aaagcttaca gacacatgca ataccactaa taaaatattg atttggagtt 4920
gttttggagg tggattttag tataggacta taaccactct cctatcttac atcagaataa 4980
acccaatttt tgtggtctag acaaaacgta atgctaagca aaaagtggag agcttgcaaa 5040
agccagagag aagacatggc gccataacta aattgatcct tgtatatctg atgcagttgc 5100
cactgcgtga gagataaagc aagttaatcg attagtatcc gatcaaaact tttcgttcta 5160
ggaaagcttt atttcgcaca catcaatgtt cttggaatgc taacccgaat cgcaattatc 5220
tgaaaccatg actagcgact tcaaggtaat aatcgtggga ggatcagtgg ctgggctttc 5280
actagcccac tgcttagaaa aaatcggtgt ttctttcatg gttctagaga agggtaatca 5340
aatagctccc caactcggtg cctcaattgg cattttgcca aatggtggac gtattcttga 5400
tcaactgggc atcttccata gcatcgagga tgaaatcgaa cctctagaat ctgctatgat 5460
gagatacccg gatggtttct ctttcaaaag tcaatatccc caagctttgc atactaggta 5520
ataacagtga aagaagagtg gcctataagt gttcatatat cgctaacttc gtgcggttaa 5580
tagttttggt tatcccgtgg ctttccttga gaggcaaagg tttcttcaga tactttatga 5640
taaactcaag agcaaagact gcgtttttac aaacaagcgg gtagtcagta ttgcaagtgg 5700
ccaagacaaa gtcacagcaa agacttcaga tggcgctaag tacttagcag atatcgtgat 5760
cggtgctgac ggggtccaca gcatcgtcag gtcagagatt tggaggcatt tgaaggaaaa 5820
ctctcaaata tcagtattag aggcaccgaa cgcaagtagg ttaacctagg attaattgca 5880
aagaaacttt actaatgagg gagccactta ggtattaagc atgattattc atgcatttac 5940
ggaatttctt taaacgttcc ccagatcatc ctaggaatac agttaaactg tttagatgac 6000
ggagtgtcaa tacacttgtt tacgggtaaa caatccaaat tattttggtt tgttatcatc 6060
aaaacgcctc aggctagctt tgctaaagta gagattgaca atacacatac agcaaggtgt 6120
atctgcgaag gactgaggac gaaaaaggtt tcagatacct tatgttttga agatgtatgg 6180
tcaagatgca ccatattcaa gatgacgcct cttgaggaag gggtgtttaa gcattggaac 6240
tatggccgct tagcatgtat tggtgatgct atccgcaagg tatgtggatg atgctatatg 6300
tccctatttc gtgtcatcag tgggatgaca aaagaaggcc actatttgcc gctaatataa 6360
atgatcgtat cgctaacatt aacagatggc cccaaataat gggcaaggag caaatatggc 6420
gatagaggac gcttgcagtc tcgcaaacat cctccagaaa aagatatcac atggttcgat 6480

tcgagaccaa gatatcaatt caatgtttca ggaattctct atggctcaac gggctcgcac 6540
ggagagcgtc tgcgcgcagt cggagtttct agtccgcatg catgcgaatc aaggtattgg 6600
aagaagactt cttgggcggt accttattcc tttcctgtat gacgcacctg ctggtttatc 6660
tggatttttct ataagtggcg caacaagaat agagttcata gacttgccca ctagatctct 6720
taggggagcg tggggaaagt catggagagg gtcatgggaa ttcatcctac aaagcttggt 6780
ctatttgcga cccaagttta ggatagttta tgccttgtat ctcgttgcag ctgcagcttt 6840
tatcttgtat tgtcttagca gtctcttccc gtagcaagga acaactgtcg aaaatggcct 6900
taatctggaa aagctaatgc ggcgatgaag gcaggcagaa ctcaaaaaca gacaagcaat 6960
gaccctcata ttgttaaatg ctagttgtta cataacttca tgtgattcga ggtgaaacta 7020
tattaaccca ttttccaact aggagaaaaa tgtgttatag aaaagtaagc aaatagctag 7080
taagaatata ataaaaagct agacatgaac ttatatttcc aacagcaaga cctaggtata 7140
tagtaactaa aaggtattac gaacctaaca tatactaata gtatataata gagtagctta 7200
tgtagaaata taagtaaaga aatagcaaat aggtaaggaa ttaataaacc taataggcca 7260
tagtagcacc atttagacta aacacaatat agttagctat agttatgtag tcataactaa 7320
gaattcaatt aagtaaacac ttagtaagat agtaataagt tactatagag aatatagagt 7380
ctatatcctt atccttgttc atagtgtcta taagctccta gagctattct agaatagcaa 7440
aacgattagc aaaattgccc tcaagtgtaa gaatagccta gtgtaaaaac catagcgtta 7500
agaaactata agactagtaa aaaaaaggga gacttgtagt cttgcaggta ttgcctctct 7560
tattacacta gatatagcgc tttaaagttt agtcttagct agagtagaaa ttaaaaccta 7620
atggaaactc aagttgattt atagtaatat agccttaata aggggttttt tttaaagtcc 7680
gtgtacttag tatgtaaata acacatatag ctacactttt caaaggaaat tgtagttata 7740
ttagtggtaa aacggtggta aatagaaggg ttaaagaggg tatgaactaa gcttaaaaaa 7800
accctaggaa agaaactagg tttataggga gaaaaaccta atcaggcaat agggaactgc 7860
aagtaaatgt tagagatagg atacttacaa aataaagggc taggaaaact ttagatcctt 7920
tagataatta agcagctagc tagctatggg atagctatgt gtttataaag caaggtattt 7980
agcaaagact acttatacta tatatagtaa attagagttt aagaccttta cacacctact 8040
cctaggtagt atctttctag tagtaactac gaatcttagc cttcaatcta ttcattaccc 8100
tataaccgaa gttataacaa atccttaaat ttttaataag tattaatcta tacttaacac 8160
atataagtac tatatttatc aagtattaat taacactata aaggttataa atataaattc 8220

tacttataaa aaggaaatat atcttcttta aaataagggc taattaatta atttaatgac 8280
gcatgaaaat attattgtta taaaggaaaa ggggggatta tttactaccc cttaagttat 8340
ataatcatgc gttgttagaa atattaaagc ttctagtgta aaataaaagc taagtgcaac 8400
taagtgtaat taaaagcact aggcttataa cctataagat agtggaaaaa gtaataataa 8460
taaattcagc tatctaagct ctttatatac gtggtataat aaggctatat aacgagagca 8520
aaagacagtc tttaccctaa gtgacaaggt ctcgtaatta gccgcgaaga gggaaagcat 8580
cgcgatgaaa gtgatgccta agatgtgagg ctgctacatc taacagatca gacccttcgt 8640
ctcctcagaa cacgcggttt gaaaagttct acctctagca actcctcgca ccaagctgtt 8700
tctacatgct cttaccgcaa tctaaactga aacccaaaat tcacctcgca catagccgct 8760
aatccgcaat tgctttaaca tgcaatacgg taatttaaca actgtattac ttctgcgtaa 8820
tactttattg tccttgaatt cttcgtcaat ctgccatgtt cactggctgc aagtgattgt 8880
ggctctgctt gtcttgatcg tctgcatctt tctatattgg cgaacaccca ctggcatcaa 8940
tgctcctttc gcaggatatc gttcaccatg ggagccgccg ctcttggttc agatgcgtta 9000
cgtcttcaac gctgcctcaa tgatacgcga aggatatgct aaggtatgtt ttatcccgcg 9060
tagaggtctt ctacccggat agaccgagaa gataacaact tcggaacagt ggaaagactc 9120
cttgttccag atctcacgat acgacggtga cattcttatt gtgcctccaa gatatttgga 9180
tgacctccac aacaagtcac aagaggagtt aagtgctatt tatggtttga ttcgggtgag 9240
gaatgccacc aaccaaaaaa cgcagagcct attagcgcat ggtctcacat attcgaattt 9300
gctagaattt tggtggtagc tatagcggca tcaccctgct tggagaaaac gatgttggca 9360
ttcgtgcgct tcaggtatgt acacccttcc aaaagtctgt tagggacctt ccttactcta 9420
ctacagacaa aaatcacccc aaatcttgcg aaattatgcg atgacataag ggatgagttt 9480
cagtattgtc tagatacaga cttcccagcc tgcagaggta tgccatttcc aaaatcccat 9540
tatgcagtct ctacttttтc tggcactaac gatatctaac atagattgga catcagtgtc 9600
cgtgcatcca ttgtttctaa aagcagtcga aaggataaca catcggattt ttgttggatt 9660
gccattatgt cggaatcccc aatgggtcca agcgaccagc aagcatgcac attacggtac 9720
gtcaattgac taataatagg caatatacgc gctcatatgc tttgcagcaa caatgataca 9780
gatagctatg agatctgtcc caaagttcat tcagccttta ctaaattttt gccttccgtg 9840
gccatggaag aacgcagcct gtgttcgtga agcaaagaat gcccttatat tagaaatgca 9900

acgccgacga aatctcgaga aagttaacag ttttgattat atcaaatcca atgacttgct 9960
gcaagcagtt atggaaatgt cttctcctag tcatgaggat agccagcttg atgttgtcgc 10020
ccagataatg ctcacgatga acacaatcgc tggccacagt actgccgcat ccggagcaca 10080
tgcactgttc gatatggtta gccactctaa gtatattgaa ttgctgcgtg aggaggctct 10140
tcaagtcttt cgacatgttg aactgcgtgt tacaaaacag gctttggggg atttgcgaaa 10200
attggacagc ttcctcagag agttagtatt gtcctaaaca tcacaatctc accacattct 10260
cacgctagct tttcctccgt actaatgatg gtcgttgcta agatcccaac gacataatcc 10320
gctaagcttg tgtatgttta gctaagagtc tcgaaaacct ggaaatgttt gtcctgtgcc 10380
cgagttctaa cgtctcttac tacagtaggc ttttttcggg tcgtattaga ccctgccggt 10440
atcacacttc aagatggcac acatgttcct tacaacacac tgctttgtgt cgcaccacat 10500
gcgatatcca atgacccgga tgtgatagaa gacccaacct cgttcaacgg tctgcgatac 10560
tacgaacagc gctgtcgtga cgccagtcaa gagaaaaagc atcaatacgc tactacggat 10620
aaatctcacc tgcattttgg ctacggaacc tgggcctgtc caggccgctt cttggcctct 10680
gatatgttaa aagtgattct aacgatgctt ctgcttcagt atgacatccg ctccccccgag 10740
agagcaaaac ggcctgtggc aggtcatttt catgagtttc cgcttttcaa tattaacaca 10800
ccactgttaa tgaaacgacg caatgattcg ctagttctat gatttattgt gactttcgtt 10860
agcatattac atagtgcgaa acttaatcta gaaaactaga gaatgaatat ctttggcact 10920
gtcatgcatg cacgccttaa catcatattc atttatatta ttactaatgg cctagatctt 10980
atttacttag tgaaactagg ggaacacatc actttctttg tcctagtgtg gttttaaatg 11040
ttattctttg cgtacatttc catatagcag cccgtttagt aaccgtattc accttgccta 11100
acaatcgttt tctaataaca cgctaagggc aacaagtgac aagtgtttag taattagtaa 11160
gcagtttagg ttagggggag caaggtagtg taagcgcagg gcgtgcggtt tattataata 11220
gaaaagaata tagtattagg gttaacacta gaaaaatccc cctagcttat taagtaagga 11280
aatagattag ataattatag tagtaatatt tatagaatcg ctctagctag cttaagtagt 11340
aattaaccat catcattacc taatcatttt ggtactatta caggcctttc cgtacagcca 11400

<210> 24
<211> 11202
<212> DNA
<213> Neotyphodium lolii

<400> 24

atttatgtct tttgcagcgc tgtcgtataa ttaagagcaa ttatggctcg ttgcagcaaa 60
caatcgccca attgatacaa tcaaaattcc acaagcgaaa gttgtgacaa ctcacgtcct 120
atcactcctg tcgtttcctt tcaacatacg gtaactgtct tctccaagcg gcgcaaaaga 180
acaacggcat tccgattggg gaggatctta tccccatgaa caatgttagg agggcgccgc 240
gacggaaact cactatctag cttaaaatca tactcgagca gaatccgact catgattgct 300
ttaatcatat actatgagca caaactcaag ggttagtcaa gaaggtcgaa tgtgcgtcac 360
atgagcttga agggagattg atatggaaaa aaaaaagatg aaaatctaca tcacatacgg 420
aagctatgaa gcgtcccgga caggcatatc tcccaaatcc gaagtgcaga ttcaaggcgc 480
tgctgttaga aaatgatgtt gtggcagtgc cagattgttt caaccatcga aaaggttgaa 540
acaagtgagc gtcggataat ttcgtactgt ccgtattgat acacttcgct ggaatcgcaa 600
tctgctgtcc cggctgtagc tggtagccat cggacaatgt aattcgttct cggacaatgc 660
gtctaaaagt gacttcaatc gccagttagt tccaagtttg acaaaagact ggcttagaat 720
agtccttaca ctcgcccact ggattgattc gttgtgactc tctcatcagg ctatccaatt 780
gtttcatatc aagcatagtc tgttgcgtga tttccgtcca accatgcttg agcacgtttt 840
gctgttctct tcggaggctc tccttcaagc tgtcgtccga gatcatgtca aaaagtatgt 900
gagttaacgc catagctgtt gtatgaatga cagccatgct caatattcct agcgtacgat 960
gcgcaagtgc ttctggccgt gcttcatcct cggtgactgc taactctgta caccactgca 1020
aaaagtcgtc gtgcttttcc tcagagttaa gtttccttct ctggacttct gaaccaagga 1080
ggttttgaat gtattgtaat tgcccgtgag cttctttcgc tgtggaaatc caccgtgcga 1140
aataatgtgt gaacaaattg ccaaaactac gggaggtgga tatcgtatca aacgtgttgc 1200
gactaaaaga agcagtagtc tcgagaaaaa tttcgtttcg acaaatcagg tctccaacga 1260
atagcctcgc tgtagcacgg gtagctagat tgagaaccaa ttcgtatgga ttgacagaaa 1320
tccaagcatt ggaacctcca ctaatattag acaacactag acaagtggaa tgtttgaaag 1380
aatgaaaaca cataccgtcg ttgccggcag gtaagacttg cccaaaggca tgattgagct 1440
catccaatag cactggaaga gtttgtgctg gattcagtta gcatagtgca aacgtattct 1500
gttagattac acttacgaag ctttggagtc aaacgctgct gtatggcacg tgtgtgaagt 1560
tcgctcccta ataatatcct cgtaacttta ccagcattgt catcaaagct ggctgttaaa 1620
gggtctaatt tgtcgctcgt aaccgcccgt aattccagcg cgtattgtaa aggaatgaca 1680
atcagatcca tgtccatctt aagtagccta aacggttgat ttttgtgctg taacctaggc 1740

ggttagtact atcatcacgg atgctcacag acttgggacc gaacaccttc tcatagccat 1800
gtttgactag gcgggcggca tggtcattat aactcagccg atcgaggaat tgggggagta 1860
atcgaggcca acgtcgaatg gttggtatac attctttccg tttggtaccg taaacaacgt 1920
accacagtag taaacagata cccactggga cagcgtgcaa cattaacatt ctcaagagta 1980
gctgatttga cttgaatgga atataaaatg atttatgaat taattttgaa tgggcttggc 2040
atctacagaa taaaagatta taagacaaat aagacaaagc ttaatgttaa aactttatgt 2100
taagtacttg aaattgtcct aagccatcga atctaatgca acgcctgtcc tcttttctct 2160
tgacactatg taaggagcct gcagcaataa ctaaggatgt gatccaagtt agcgcagtta 2220
tttaaaattc gtcattttta gacccactag cgccttccta tatttagaca gtattttacg 2280
gtatataacc tagataattt caccccttgct tataatacaa tacaatcccct gaattgtttt 2340
acaaatctat ataatagaag taattgagct aattaaatta tagctaggaa ataaaggaga 2400
caggggtgg tatattttag tactagaacc tgcatagaaa tagatattct cttttgtgac 2460
gctatatacc ttgcatattt cccttgtagc tctctaataa taggattact tatagctaat 2520
cacagccgtt agggaggaat caataactag ggcatgtaga cttgtaaaat aattcagcgg 2580
gtagagtgtg tacttaaatt acagtggtgt tacaggggct atttagatag ccaaaagagg 2640
gaagccctat ttctgacact ggcgtagtaa aaaaaaaaag tgcgctaatg tattacttta 2700
ttcttacgga ttagtatctg atcctattgc aggcatttac ttggcactag ttgaaaagat 2760
atattataaa caggggggag tggttttatg caatgtgaac aaagtttcac aaatttctac 2820
tccgtataaa cataatttat tgggggtctt gacatgtccg tcttagccga caaccccacc 2880
atgccacgaa tcccgcggag accccaatca atccatacac ggcacttaca cagatcattc 2940
catccgccca agtaccgtcc tatactccgt acaccctaaa agccttagag cacgaacata 3000
tgccgttgtc tccctagtca cacacaagat gcctaccccc cttcccgatt ccccttctca 3060
catgtgtaac gtatgtaacg caaggttagg tcgcggtggc acaaaaaagt aacgccgcag 3120
ccgaaagcca tcctgtcgcc agcggaggtg tcgcggttcg tatctgctta gctgtgtttc 3180
attgttaggc gtgtgcataa tgcgcggggt gcgtttaaat gtctagctga agtcatatct 3240
gttgccgtgc atcacatcac ttttacttcg ggcaccattt catgcaccct aatagccacg 3300
acacacagaa tccatcacca attaactcag gcagttcgca cctacactaa gccattcgaa 3360
caatatacat tacttcaaag actcaccttа ggccgtcttt tcacgcagcc aagaagtttg 3420

aacagctcgt tcctacatcc ctgcgaatcg gacgtttttc acggatagac cctctaggcc 3480
cttaatgaag acttctaaat gtcaggagct atctaagtca agtacgttga caatacattt 3540
ctttggaagt gttgtcttcc gttttcttat ccctcttatc cctttagcct aggttttcta 3600
aagttaaagt cgtcaagcta ggttcgatat gaagatgtta acagagcatt ttgactttcc 3660
taaacttaac ttcgccacca ttgtaatttc aggcgccacc attattggta taatattcct 3720
tcgatatctt aattacccta caaaggtttg aatctgtttc ccttgctatg cacttttctt 3780
ggatgctcac caaaattttt caaggtgaat gtacctgttg ttggcattgg agttcgatat 3840
acaaaatggc tagcggctat tataaacgtg cgtcatgctc gacaatctat ccgcgagggc 3900
tatgcaaagg tttgtgttaa aaacgaataa aagcgcttcg taaacaaaga gaactaatac 3960
tagtttctag tatggcgatt tcgcgtttca gatacctact atgactcgaa tggaggtatt 4020
catttgtgat agacagatga caagggagta tcagaatgtt gacgactatc atttgtcgtt 4080
ccgagctgtc atgaccgagg taagtaacta gaccatgtta actgtaggaa aagaagaaaa 4140
agctaaaccg ccgtacagga gtttcaattc aaatggctac ttccaggaca ggcacacgaa 4200
gcccggatta tccctaactc agtgattgct aaggccttga gctggcagag aacaagggcg 4260
aataaaccca gcgatccatt cttcgaatct ttctccgccg aattcatgca ggggtttcag 4320
gaagagatgc gacgactaat ccaatatcaa aattcgtcag ttatgtcaaa ccgctccggt 4380
gctgtcctgg atccagcgca tggttggcat gctgtgcctt gttttccctt ggctctgaag 4440
gtaattgggc gccttactac atacgtcttg ttcggcaaac ctttgtgcca agatgcgaca 4500
ttcctaaaca tgtgctgtca atttggcgat gtgattccca gggatgcgat catactacgt 4560
tcatggccag cattggcaag gccgtaagca agtgcctaga cataaacccg tcagggttta 4620
aactcgcatt aacattcata tagtcttatt gtaaagatct tgagtgctcc aagggttatg 4680
ggaaagttgc gaaacatttt gattgttgag ataaagagca ggagagaatc ccacgaaacg 4740
aacccaatga gtgtatggct gtctcgcaca ccccctctag cattacacat taacgtatat 4800
ctaggatatc ttggatttca caatggcctg ggttgaccgt catcctaacg ctagctttga 4860
cgatcagcac attgccgaga tgatgattaa cactattttc gcagctcttc atacgtcgag 4920
tcaggtatat ttttttctgt atgaaaagtc cagagcttaa agctaactgg ctcatagctg 4980
gtggtgcata ccatctttga gcttgcctca cgtcctgaat atagcgatgc gcttctggaa 5040
gagatagatg catgctttga aaagcatgga aagggcacta aagcagctct agactcaatg 5100
ttcaaggtgg atagtttcat caaagaaacg cagaggttta accctcttga cgcatgtata 5160

aattccctgt ctccgattcc atcattgcga tttgactaac gccaccgtca gccgctcttg 5220
caagactggc tctcaaagac tttacttttt ccaatggcct aaacatccca aagggcagtg 5280
tgattttcac gccgaattcg cctatctttg aggacgagag atattacaag gatccgaaag 5340
tttttgatgg atttcggttt gctaggatgc gtaatgaccc aaaattaggt ctattctgcg 5400
acctaacagc aacgaatgaa caaagcatgc attttgggac tggacgtcac gcctgtcctg 5460
gtagatttat ggtttctgat gaggtcaagt tagctgtgat tcatatctta agtaatttcg 5520
atttttgtat tgagaatttt ggaccacggc cagcaaatca gccatttggt aaatttcttc 5580
tacctgatat gagtgcaaaa atctggctaa gggagaaaag agctagggag aagaatctgt 5640
gaaagccgtt aagataatgc caattgctac acgatacata tatgttcatg ttagcgagtt 5700
ttgaagagaa gctttgaggc ctctaagaaa ttttaactac ctatgataat gaagcagctt 5760
tatttctaac atgatttttc tagcctgtga aaagtgattt ttgcagctta gacaatagga 5820
tacatgttat tagcctacct gaggggggcta tggtaagtga ctctaagatc tcgcaatatc 5880
aatgaaacta taggcaatat ctagctaatt aggcctatat ctatgcttac aaatgcagta 5940
ttacctctaa gtctatagat aacaagcata cagctagttt cgtttcatta cgtaacggtt 6000
tgtctctaag tagcgacagc taagtgagac agatatatca ggcacaatac aatacacccc 6060
ctgagttctt ttacaaatct acatgcccca gttattcgtt cctctctaat agctatgatt 6120
agctatatgt aatactatta ttagtgagct ataagggcaa aatacaaggt atataacgcc 6180
ataaaagagt atatttttt tttctctatg taagtgccta gtacaagaat gtactatccc 6240
ctattacctt catttcctat ctgttatcta attagcttaa ttaccctgt tatgtggatt 6300
tgtaaaataa ttcaggggt gtattgtaca tcattccaat ccgtcttaaa tcatagatat 6360
atgctccttg ggctttcgtg ccacaccccc ataagtacaa atgcactgtt cacatgtttc 6420
acagcctttg attgcctaga agagacgaat aggtataata gtgcacatat tgccatccac 6480
ttaatgctaa tatccctttc gctcgctctc tttatctttt gtggacgcag gtcctatttt 6540
tcacatataa gcacttccga acctgcgtaa aatcttactc acgcaaggaa atacaattca 6600
aattatatcg tgcttgattg atctctttct aggcttcctt ttgttcaaga gactaactaa 6660
caattgttgt cttcggctct cacattacac catgattgcg aaaaatattg aactcaatgg 6720
cttggatccg gcaaccaggg cattggacat tctatactgg aaaaatcact gcatcaaaca 6780
gctagaatct ctcctatgcg ccacagattc atactgcact gcagacaagg ccgctcaact 6840

acgcattttg tcagagttgg tgctccccaa tcttggccct cggccgtcca atgccactgg 6900
gccatcctat cttacacgaa gtggttcccc aataatgtta agtctaaata caacatcatc 6960
aaaaaactgc gtcagatatt gctgggagat tctaggggcg actggcgcaa gtaatgatga 7020
tcctttggca gtccaagttg ctaaggatgt agtggcttct ctgtctgcta cttttcgcct 7080
ttcaacaaaa tggagcgaaa ctctactgtc caattttgca gtaacaccag accaagctcg 7140
acaagttatt aacatgctac ccgagtggat tcaaggcttc gtacctgagg gaatggagtg 7200
cgattttcca aagagaatcc cgttcgccat gacatcattc gacctaaatg gctccaatgt 7260
agctatgaag ctctacgtta atccaagggt aaaggagatt ttaactggta ctccctcatc 7320
agacttggtc tgggagttcc tccgaaattt aacaccagaa atgaaaccac gagcggtcga 7380
cttgcttgag aggtaagaat ggctttgaac tttcgcccac cttgtcagcc ccatacgcta 7440
agcgctaact ccccacacat taacaggttt attaccgata attcaggccc gtctgctatt 7500
gagcttgtag gtattgactg cgttgacgac gctcacctat caaatgcaag ggtcaagctt 7560
tacgttcata ccatgagcag ctcatttaac accgtaaaga attatgttac tcttgggggt 7620
gcaatctggg atgaacaaac ccaaaagggc ttaggaatac tacaaagtat ttggcaccta 7680
ttgcttcagg agccagaggg tatttctgac aatggattcg acaagcctgt gaacgactct 7740
tccatgttat gccaaaagct atattttagt ttcgagctac gcccaggtac agacttccct 7800
caggtgaaga cctatgtgcc aacttggaac tatcttcgaa ccgacgggga aactatccag 7860
aactatgagg cgatcttccg agcttgtgac catccttggg gtgaagatag gacgtacggc 7920
aaaatttttc aagatgcatt gtaagttatc ccttcagatt agcgctaaaa ggagtttgag 7980
atactcctca atgcaagcta ttaggttgtg aaattgccac tactaattgg agctttttat 8040
agcggacctg caaccgagag tcggaaaaaa cccattcact gcgacgcatc ttttctgttt 8100
accgaagaaa ctggtgtcta ccagacgctg tatttcagtc ctccgattga gggggaaaca 8160
gaagtccagt caaatctcgt tgcttgaggt tgaattaact ccgcaatgct acgtctaaaa 8220
gaagtgtctt tggtgaacag atgatagggt tcccttgatc tttcatatat ttgtgtacag 8280
ctgtggaaat ttagggtcta gctctagata aagccattgc ttcaatcgtc atttgacgta 8340
ttctgagtct tgagctattc catatttttt ttctaattaa tcttgacttt attaagtgtt 8400
gtaggccgtt gcaataatat ttgctttgat cttacaagtg tagcagctac ccttgcactc 8460
ttcgattctt gaacgagcgt tgctattcgg agctgtgttc aagaactagg ttgtgcgcat 8520
aggtttaatt ttgcaatatc acgaggagag gcccgttagc caactgctta aatacaggtc 8580

ttgctagaaa atggttgcct taatacagct gctatgctac ctcctatctc cttaagcgtg 8640
ttctaccttg tgggtctagg ctttggtaaa gggtagttat tacaggcaag agatgtcaca 8700
tcaagatagt ttttgtctag catagcgcgt gagttacatt tctccgaaat cattttgtaa 8760
agtgcatttg ttcttttcgt ccccagacgg ccaccaagtt ctctagaccc tgacctgcct 8820
cctggagcgt tttgagacac gtgtttttaa cactaggtga ctctaacgct tggagtgccc 8880
gtttaatgtc gccttgattt tggctctgaa acgccttcct tatctgcggc tcagtatgtt 8940
tgtcattaag agcaaccaca acaggaaagg acaattctct gttgcgtagg tcttctgcaa 9000
cagtgccttt gttaaaagcg tactcttctg agtatatgtt cttgcaatca ttttgcaatt 9060
gtgcgtacca gctaccattc cattgaatta gttaagcgac cttgccgcgg aattatgaaa 9120
cgagcgaaaa aaaaaaaaat ttacataccc aaatcggcct aacagatcgt ctgattgatg 9180
acctccttgg ttcaaaagcc tcccaaggag cacaaaaagt gtgcctgtct tcagcagagc 9240
catatttttg tacgtcaaca ggctttcttc accataggat tcgaaagatc gcaaaccgtc 9300
tcttcgccaa accaaagaca tgtcttgtcc ctcgagtatc agttctagtg ctctcagaag 9360
ttcaattcca aggacaggtt gttctttcat tgctctatta atgacctttg tgagaacgaa 9420
gtatgctcga ttggcagttt cgcatgatcc gtatagcaag tgagccgtag tgtgattttt 9480
ccgcttaggg ctgtggtcgc aaatatcgtc aaccataatt agacaaagat ggaccgcatc 9540
catgatatcc aggatcaaag aatgtttctt gggatattta tgtttcaatg ttggatgaag 9600
aaaatccacg aggggagaga aatgattatg cccatagaga ctaatcacat aagagtatgg 9660
acagtcagga atcttgccat tgctgccgcc aataccttcg tgagaggtac gttcatagcc 9720
atagccataa tgatcaatgg cttgagaaac gacagattgg taattgattg tgtactttac 9780
tagatacccg agcgtaaatg caaacaagag ggccgcaatt tcgatggccg cagggcgagc 9840
cacgagccat gctccagatg tcatcttgga atagtgatgt acgtcggcta aaggcagatt 9900
gccttgagaa agaattcagt taagcaaagt tttatcaatt ctcgcaatat atgcagagca 9960
actcaagcaa atgttaggaa tatcgttaga ctataattat agaggcagag cttctagaat 10020
agcgcaatca tagtctatta tgtatacgcc caggcgcggt taaatacata catataattc 10080
aacgaccttg tcaggcaatc aagatgtgct actcttaatt acataatgaa caagatgcta 10140
gaggtattaa aggccaaaat gtgtccttct ggatagcaga ccggactaaa ccttcgcaaa 10200
ccattcctat aatactagct gattttatca ctatggacgg attcagcaat atggagcaag 10260

cgccgctcgc ttatcaggaa gttcaatggc tagctgaaac ttttgtcact ttcatggggc 10320

ttggctggct tatcaattac gtcttgatga tctggcactc taggaggggt gaaccgagca 10380

gcatggctct catacccctc tgcaacaaca tcgcctggga gctcgtatac acgattatct 10440

atccgtctcc taacaaagtg gaacttgcgg ctttcatagc aggtgtcact ttgaacttcc 10500

ttatcatgac ctctgcagcc cgttcggcaa gatccgagtg gagtcactca cccacaatgg 10560

ctaagcatgc aggtttgatt atagtcgcag gaatattgat gtgcttcacc ggacatgtag 10620

cattggcgat ggaaatagga cctgcgcttg cttactcatg gggagctgtc atatgccaac 10680

tagctctaag cattggaggc gtgtgtcaat tgttgcagca gcatagtact ggtgggacat 10740

catggaaact ttggtaagtg aataaatcaa ttacgtttct aatctatatt gaatgtcata 10800

tcaggggtgg ctgacatgaa agttttcagg tcaagtcgat ttctaggctc ttgttgtgcg 10860

gttggctttg cctttcttcg ctggagatac tggcccgagg cgtacggatg gctggccagt 10920

ccccttatcc tctggagtct tgccacgttt cttgtggccg atttgacgta cggggtttgt 10980

ctccttcttt aggcagaaga gaggacagtc gaactccact aagcttcaga ctgcgcaacg 11040

aaaacgagta acggcttgag actagttcta tcttatcgat cgctatctta catggttaat 11100

gtaaccttct atctttgtct aagggcttac actcaaatga aatcatacat gcaacttaaa 11160

ctatcataca taggagagtg ccaatttaag caatttaagc ac 11202

<210> 25
<211> 6583
<212> DNA
<213> Neotyphodium lolii

<400> 25
aagcttttta ccctaaatta tagtataaaa aagcaaatct ctcttagtaa gctactttat 60

aatattaata tatatatact attactctta attatctagt ataataataa gtaaataatc 120

tagattacta aatatataga aaaaaggctt tagactagcc ttaagtacct taatataaat 180

taaatataga tttaagaata atatataata ggtaatatta agattaatta aataaaaacc 240

ttagagatac ctatagatag ctttataaag ctacttttag gttaaacata taaggacttt 300

tactataaaa ttagattata agaaattcct atttaattat aatattaggg attatttata 360

atctcttcct ataccttaa tctttagctt taataaatat cttagctagg gggggtatat 420

taggaaaagc tccctaaagt aataaacata taaatatagc ctatataata actaggttaa 480

aacccttaat aaaataatag taaagataat atattagaag tatctttata gactagttat 540

tactatagcg cactatatac cttttaggta taaaagttag gtggtgttta cacctctagt 600
taaccgaaat cgaacctttt ctattatcta caccgacccc taccttgaca gttaggactc 660
ttgttcctgc aggtcagcta gcttgagcgc aaggcaccgt tgcaccatgc atgtcatgtg 720
cagcacggcg catagatgcc gatgatgccg ccagtgccta tcttgaaccc cagatgcaga 780
ccttgatatg gcctcttgcg agtaaacgca ttgcgactat acggccttga agggcattga 840
gttggaggct cttccctata atatgtgctt gtagaattgg tatcgctgct tcattttaag 900
cattggaaac attcaggaga cgtactacgc ttgtgtatgc aacgcttttc tttttgaaca 960
atgaaaccta ctactcgctg tccattcgac tatctggtga gccagtgtgg aaagcatcat 1020
ttcaaaacct ttgtccagtt actgtcacct cttctccaag acgaagatcc cgacagatat 1080
gctttaattc tggacattat ggacgctgtc cacttctccg ccatcttgat cgatgacatt 1140
gccaaccaaa gtgctctacg caggaaccag ccggccgccc atgttgtttt tggggagacg 1200
gaaacggcca ctagagccta cctcgttctg ctgcgggtcg tcaacaggac aatgcgagag 1260
aacccagtcc tggccggtga gctactaaac agcctggaag aaattcacca gggtcaggac 1320
gagtctcttg tgtggcgccg cgacgggctg gagacttttc ccgtcgccga tgacgagaga 1380
ctggcagcct acgtgcgcat gtcccgcctc aaaacgggct ccctcttcgt gctcctcgga 1440
cggcttctag ccaacggtgg taccgagttt gatgacctgc tggtacgctt tgggtacgtt 1500
tctgcctctt gtcccaacaa agagggccgc cgactaactg gttctctctt gttcttgaag 1560
cctgtacgcg cagctgcaac atgattgcaa gaacatttac tctcccgaat acgctctcaa 1620
caagggatcc gtcgctgaag atttgcgaaa tggcgaattg tcctatcccg ttgtggtcgc 1680
cttgattgag aacaaggcgg aagggatcgt gggagaggcc ttgcgcacgc gcagcgacgg 1740
ggataccgaa caggcactcc gcgttttgga gagcccggca gtcaaggacg cgtgcctgca 1800
cgcactcgag gctgcgagtg tcggcttgga agaccttgtt gaggcatggg gacgacgaga 1860
aaaaatgaga tccgacaccc tcgacggcga cgacttaaca aggccaagca ccatcacaca 1920
acatgaacaa gatgaccatg ttgatagagc tgccatcgat gccaagagtg atgcgagtgg 1980
cagtagcaat aagtctctca cgcccccaga gacagccccc acgacggaca ccctgtccga 2040
gacagctgtg ggggatatct cgtcagtcga cgtggattac tggactcgga gatgcgttcc 2100
cataatcggt agcctcttga aatcatgccg agtctactcg gaagcggaac gggaaacaca 2160
gctgcgcttc cttcaggaac atgtgctacc taatctaggt cctcgtccat cctcccccgg 2220
ctcgcagatc cagtccatgg ctacattcag cggctttcct ctccaaccca gcatcaacct 2280

gagcggctcc ggccaggcca aagtccgcta cacgtttgaa ccgctcgaca gcctgagtgg 2340
caccgaggtt gacccttttg cactggcgcc ggctcagcga gtgctcgaaa agctctccac 2400
ccttctcggc gtctggcctg gatggatcga cgctttgatc gctgcgtacc acccaaccag 2460
agaggaagtc gagcaattac acccgaatct gcacgagtac ctcagaggcg tcctcgtgag 2520
aacgacagga cgccaagatg tgcaggttcc tcccatgccg cgaatgtggg tgtgcttcgt 2580
tgcgcttgat ctggagggcg cctcacaggc actcaaggtt tattttgatc ccaagatcaa 2640
agaagccgtg actggtattc cttcctgcaa atacacttgt cagattctac ggacggttga 2700
taggtttggc aacgccaagg ccgtcgacat gcttgagcag tgagtcacgt ctgggagcac 2760
tatccaggtc cgaggtacta acaagatttt gtcaggttct tggcagagga gcacagcata 2820
ggcgctgtcg aactgattgc cattgattgc gtcccagaag aaatgcagcc atcggcgcgg 2880
atcaaggtct acgttcacac catgagcaac tcgtttcaga cagtacgcaa gtacatgaca 2940
atgggtggcc gctgcatgga tcctgcgacc ctcgagggtc tggaaaactt gcacgacgtg 3000
tggtactccc ttctcgggga aagtcaaggt attgtcaatg aagagtacag caagcccttg 3060
actggcttta gctcgatgca gcatcacttg tactttagct acgagatgac gcctggcaat 3120
gctgatcccg gcgtcaaagt ctacatacct gtgcaaagct acgcgccaga cgacaagacc 3180
atcgcgcaga actacgaggc aaattttcgg caactcaact ggccgtgggg cgaacccggc 3240
gtttacgaag cggtgataga gagtgctctg tacgtaatga caggcccttt gaccatatta 3300
cttactgaca acttggaatt tagtggacca gtaaagcaca gccgcgcaac gttcctccat 3360
ggaggatctt ctttcatctt ttccaaaggc cgaggagttt atcagtccat atatctagac 3420
cctccactgg aggaaggagg gaacattgct gtattcgagc accacgacga tcaggatact 3480
atagttgacc ttggcaatat gtagtcttgt catcaattga tcagctgtat gagctcttgt 3540
gtttttttcct ttagctagtt tggcctgaat gtttgaaaaa catgtctgta tgaactagtg 3600
atggaagaag ggttgaaagt gagcatgtac cgcaaaacat tattccttca ccttgctcga 3660
gatagctcac cgtaaacgta cctggtgagg tattccagaa ttgagccggt tcatttcgc 3720
ggacatgaac ccatcatcag cttttgccga tcctaaagtc tagacctgaa tagtgacgca 3780
gctggtatga ttggtgcagg acaattactg cgcccacggc ggacgcgatg ccgggggcc 3840
gcccggagac cccggcatgc agaacgatca gctcttgagc tcctacgtcg cgcatgtctc 3900
attcaagcat gcactatata ttgagaccta ctgtatgcag cctcgaatgt aaccgtagta 3960

ttcaaacaag aaacatgcat atatttgcat gatgctttcc gtggcgctgc gcatatgata 4020

tacatggttt acatatgagc tgacttgaag cacggcatag ccggaggatt cttctgcatg 4080

gagcactgta tccggctgaa aattacattg tacgaggtct caatctgcgg ccagctagcc 4140

gagcaccgga gaaccggcgc atctctgctt gaactcgggc aagggactca cttctacaaa 4200

agtcagagat gcataccatc aactgaaatc aagttaggat ttatagcctt tatatttcag 4260

tggcatcact caattacttg tttgaactac gccgtcagag gttcacctac taccagaaac 4320

gacagcacca tggcatttgc aagtcttttg caccatatct ggaaccatgc agtggattgc 4380

gctgagcagc tgacttggtg gcagaccatt gtgagcttca tcattttctg catcatgtgc 4440

tcttggctac ctgggaatgg ggaaatgcgc gctccgtttg ttggttatcg ctggccattc 4500

gagcctactt tctgggtccg aatgcgcttc atctttcaga gtttaggcat gatgaccgaa 4560

ggatactcaa aggtgagctc ccgtccgggt ggagaaagac agctagacga atgactgacg 4620

ccaaacgctt gacagttcaa ggattccatg ttcaagatca cgaccaacga tgccgactgg 4680

cttgtcctct cccaacgcta cttggatgac ttgcagtctc tgccagccga gagattgagc 4740

catacagacg ctctagtgac ggtgagggcg catactagtc gctagtccct acgacagtgg 4800

tgtgctaatc gagttgtgtc tcatttagat gtgggggagc agccacagcc cttttgctct 4860

gctcaacaag agtgatctta gctctcgagc tcttcgtgta aggaccaatc cctccttgtt 4920

atgcagaacg gatctgactt gaaaaggacg tggttgcgcc gaattatgcc aaggaccttg 4980

atagcctcgt agacgaactc cgctattcgc ttgagcacga tatagacata caggatggta 5040

tgtatgcgcc tattttccaa ctaattttga ggtcgtcatg ttggctgact gggtcgatgc 5100

gcttagactg gaaaccgatt gatgcccttg aactttcttc gaagttggtg ttgcggatat 5160

cgcagcgaat cttgatcggc tggcccatga gtcgcgatca agagctcctt gaatgcgcac 5220

aaggctacgc agacgctggt aagaggacga gctgttacgt atgacccttt tcttcggtaa 5280

aaactaacgg gggtttcagc taccgtcgtc cagtttgccc tgaaactact tcctcgccag 5340

attcggccgc ttgtctatcc tctgctccca caagcatggg ctactaaatc gtggatcagg 5400

cgctgtgaca agatactggc aaaggaaatg caacgtcgac aagttttgga gaagtcggat 5460

cccgtgtacg agaaaccaaa ggacttgctg cagggcatgg tggacctgga gccgtcccgg 5520

cctgttgaca aacttggaca tgattttctc gtccaagcct tgatttccag aatggctcca 5580

gttgttacca tggcccaaac ccttgttgat cttgccctcc atcctgagga tatcgaggag 5640

ctgcgtgatg aggttctgca agtcatagga ccagacgggg cgggattagg aaacctacga 5700

caatcattta ccaaacttga caagatggac agcgtcttga gggaatctgc caggttcacc 5760

cctctatcta tgagtaagtg ccatttctgt cctccagaat agcttgctgg catgactaat 5820

ctgtggtata gtgacaatgc accgccgggt tcaggacgcc aagggcatca cgctccatga 5880

cggtgtgcat cttccacgag gcacgcatgt ggcattccca gcgtaccaca ttggcagaga 5940

tcccaagttg gtgtcaggtg cagatatcta tgacgggctg cgctggtaca ggaaggacct 6000

cggcgaggcc caagaaaacg aagctcccaa gcatcgattt gtcacccccg acagcaacta 6060

cttgaccttt gggtccggta aatacgtctg ccccggccga tttatagcgg aacacatgtt 6120

gaagctgatg atgaccgccg tgctcctgcg ctacgagttc aagtggcctc cgggagtccc 6180

tgtgcccgaa caacagtatc ggcatgtctt tgcttatcca agcaaaacca cactgttgat 6240

taaacgacgc aaagatggcg atcagattct ttaaagtatc attatctgaa aagaagaaaa 6300

gaggatgtct tcctcttccc gttaaagact gctgagtgca agtttgtgaa aggagaggtg 6360

ttacgaacag aatgtacatg cccactagaa cgagttagag tatggcagct accttgacta 6420

atatgttaac tttaataata tataattgat tattaattgt ttttaaatat ttagtattta 6480

ataaaaaata gaatattgta ttttatataa attataatta aacaatatat tatgtttaat 6540

aatataatta aatataaaat acttttattc aagattataa aac 6583

<210> 26
<211> 20
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<220>
<221> misc_feature
<222> (6)..(6)
<223> n is a, c, g, or t

<400> 26
caymgnggtc arggtatgga 20

<210> 27
<211> 23
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<220>
<221> misc_feature
<222> (15)..(15)
<223> n is a, c, g, or t

<400> 27
ttcatrtagt cgtcncktat ytg 23

<210> 28
<211> 23
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<220>
<221> misc_feature
<222> (12)..(12)
<223> n is a, c, g, or t

<400> 28
aactttccyt cngtsargtc ytc 23

<210> 29
<211> 24
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 29
gcttggatcc gatattgaag gagc 24

<210> 30
<211> 24
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 30
ttggatccgg ttcccggtcg gcat 24

<210> 31
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 31
tggatcattc gcagatac 18

<210> 32
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 32
gtgtgagatt aagacgtc 18

<210> 33

<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 33
accgacgcca ttaatgag 18

<210> 34
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 34
actgggcatc ttccatag 18

<210> 35
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 35
attagaggca ccgaacgc 18

<210> 36
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 36
atcaagctgg ctatcctc 18

<210> 37
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 37
aaataatggg caaggagc 18

<210> 38
<211> 19
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 38
tgggaatttt ggaaatggc 19

<210> 39
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 39
gctccttgcc cattattt 18

<210> 40
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 40
gtcttgatcg tctgcatc 18

<210> 41
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 41
tgtccgtgca tccattgt 18

<210> 42
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 42
catagagcta gctagagt 18

<210> 43
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 43
gttcggtgcc tctaatac 18

<210> 44
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 44
gaggatagcc agcttgat 18

<210> 45
<211> 24
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 45
gattggtacc ttgaagtcgc tagt 24

<210> 46
<211> 25
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 46
gtagggtacc tctagtactg cctct 25

<210> 47
<211> 18
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 47
tagcgaatca ttgcgtcg 18

<210> 48
<211> 20
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 48
atggctgcca atgactttcc 20

<210> 49
<211> 20
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 49
aggccatttt cgacagttgt 20

<210> 50
<211> 20
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 50
ccagcaagca tgcacattac 20

<210> 51
<211> 20
<212> DNA
<213> Neotyphodium lolii; Epichloe festucae; Epichloe typhina;

<400> 51
tgcgtgagag ataaagcaag 20

<210> 52

<211> 2544
<212> DNA
<213> Neotyphodium lolii

<400> 52
atgaaaccta ctactcgctg tccattcgac tatctggtga gccagtgtgg aaagcatcat 60

ttcaaaacct ttgtccagtt actgtcacct cttctccaag acgaagatcc cgacagatat 120

gctttaattc tggacattat ggacgctgtc cacttctccg ccatcttgat cgatgacatt 180

gccaaccaaa gtgctctacg caggaaccag ccggccgccc atgttgtttt tggggagacg 240

gaaacggcca ctagagccta cctcgttctg ctgcgggtcg tcaacaggac aatgcgagag 300

aacccagtcc tggccggtga gctactaaac agcctggaag aaattcacca gggtcaggac 360

gagtctcttg tgtggcgccg cgacgggctg gagacttttc ccgtcgccga tgacgagaga 420

ctggcagcct acgtgcgcat gtcccgcctc aaaacgggct ccctcttcgt gctcctcgga 480

cggcttctag ccaacggtgg taccgagttt gatgacctgc tggtacgctt tgggtacgtt 540

tctgcctctt gtcccaacaa agagggccgc cgactaactg gttctctctt gttcttgaag 600

cctgtacgcg cagctgcaac atgattgcaa gaacatttac tctcccgaat acgctctcaa 660

caagggatcc gtcgctgaag atttgcgaaa tggcgaattg tcctatcccg ttgtggtcgc 720

cttgattgag aacaaggcgg aagggatcgt gggagaggcc ttgcgcacgc gcagcgacgg 780

ggataccgaa caggcactcc gcgttttgga gagcccggca gtcaaggacg cgtgcctgca 840

cgcactcgag gctgcgagtg tcggcttgga agaccttgtt gaggcatggg gacgacgaga 900

aaaaatgaga tccgacaccc tcgacggcga cgacttaaca aggccaagca ccatcacaca 960

acatgaacaa gatgaccatg ttgatagagc tgccatcgat gccaagagtg atgcgagtgg 1020

cagtagcaat aagtctctca cgccccccaga gacagcccct acgacggaca ccctgtccga 1080

gacagctgtg ggggatatct cgtcagtcga cgtggattac tggactcgga gatgcgttcc 1140

cataatcggt agcctcttga aatcatgccg agtctactcg gaagcggaac gggaaacaca 1200

gctgcgcttc cttcaggaac atgtgctacc taatctaggt cctcgtccat cctcccccgg 1260

ctcgcagatc cagtccatgg ctacattcag cggctttcct ctccaaccca gcatcaacct 1320

gagcggctcc ggccaggcca aagtccgcta cacgtttgaa ccgctcgaca gcctgagtgg 1380

caccgaggtt gacccttttg cactggcgcc ggctcagcga gtgctcgaaa agctctccac 1440

ccttctcggc gtctggcctg gatggatcga cgctttgatc gctgcgtacc acccaaccag 1500

agaggaagtc gagcaattac acccgaatct gcacgagtac ctcagaggcg tcctcgtgag 1560

aacgacagga cgccaagatg tgcaggttcc tcccatgccg cgaatgtggg tgtgcttcgt 1620

tgcgcttgat ctggagggcg cctcacaggc actcaaggtt tattttgatc ccaagatcaa 1680

agaagccgtg actggtattc cttcctgcaa atacacttgt cagattctac ggacggttga 1740

taggtttggc aacgccaagg ccgtcgacat gcttgagcag tgagtcacgt ctgggagcac 1800

tatccaggtc cgaggtacta acaagatttt gtcaggttct tggcagagga gcacagcata 1860

ggcgctgtcg aactgattgc cattgattgc gtcccagaag aaatgcagcc atcggcgcgg 1920

atcaaggtct acgttcacac catgagcaac tcgtttcaga cagtacgcaa gtacatgaca 1980

atgggtggcc gctgcatgga tcctgcgacc ctcgagggtc tggaaaactt gcacgacgtg 2040

tggtactccc ttctcgggga aagtcaaggt attgtcaatg aagagtacag caagcccttg 2100

actggcttta gctcgatgca gcatcacttg tactttagct acgagatgac gcctggcaat 2160

gctgatcccg gcgtcaaagt ctacatacct gtgcaaagct acgcgccaga cgacaagacc 2220

atcgcgcaga actacgaggc aaattttcgg caactcaact ggccgtgggg cgaacccggc 2280

gtttacgaag cggtgataga gagtgctctg tacgtaatga caggcccttt gaccatatta 2340

cttactgaca acttggaatt tagtggacca gtaaagcaca gccgcgcaac gttcctccat 2400

ggaggatctt ctttcatctt ttccaaaggc cgaggagttt atcagtccat atatctagac 2460

cctccactgg aggaaggagg gaacattgct gtattcgagc accacgacga tcaggatact 2520

atagttgacc ttggcaatat gtag 2544

<210> 53
<211> 788
<212> PRT
<213> Neotyphodium lolii

<220>
<221> misc_feature
<222> (185)..(185)
<223> Xaa can be any naturally occurring amino acid

<400> 53

Met Lys Pro Thr Thr Arg Cys Pro Phe Asp Tyr Leu Val Ser Gln Cys
1 5 10 15

Gly Lys His His Phe Lys Thr Phe Val Gln Leu Leu Ser Pro Leu Leu
20 25 30

Gln Asp Glu Asp Pro Asp Arg Tyr Ala Leu Ile Leu Asp Ile Met Asp

35 40 45

Ala Val His Phe Ser Ala Ile Leu Ile Asp Asp Ile Ala Asn Gln Ser
50 55 60

Ala Leu Arg Arg Asn Gln Pro Ala Ala His Val Val Phe Gly Glu Thr
65 70 75 80

Glu Thr Ala Thr Arg Ala Tyr Leu Val Leu Leu Arg Val Val Asn Arg
85 90 95

Thr Met Arg Glu Asn Pro Val Leu Ala Gly Glu Leu Leu Asn Ser Leu
100 105 110

Glu Glu Ile His Gln Gly Gln Asp Glu Ser Leu Val Trp Arg Arg Asp
115 120 125

Gly Leu Glu Thr Phe Pro Val Ala Asp Asp Glu Arg Leu Ala Ala Tyr
130 135 140

Val Arg Met Ser Arg Leu Lys Thr Gly Ser Leu Phe Val Leu Leu Gly
145 150 155 160

Arg Leu Leu Ala Asn Gly Gly Thr Glu Phe Asp Asp Leu Leu Val Arg
165 170 175

Phe Gly Leu Tyr Ala Gln Leu Gln Xaa Asp Cys Lys Asn Ile Tyr Ser
180 185 190

Pro Glu Tyr Ala Leu Asn Lys Gly Ser Val Ala Glu Asp Leu Arg Asn
195 200 205

Gly Glu Leu Ser Tyr Pro Val Val Val Ala Leu Ile Glu Asn Lys Ala
210 215 220

Glu Gly Ile Val Gly Glu Ala Leu Arg Thr Arg Ser Asp Gly Asp Thr
225 230 235 240

Glu Gln Ala Leu Arg Val Leu Glu Ser Pro Ala Val Lys Asp Ala Cys
245 250 255

Leu His Ala Leu Glu Ala Ala Ser Val Gly Leu Glu Asp Leu Val Glu
260 265 270

Ala Trp Gly Arg Arg Glu Lys Met Arg Ser Asp Thr Leu Asp Gly Asp
275 280 285

Asp Leu Thr Arg Pro Ser Thr Ile Thr Gln His Glu Gln Asp Asp His
290 295 300

Val Asp Arg Ala Ala Ile Asp Ala Lys Ser Asp Ala Ser Gly Ser Ser
305 310 315 320

Asn Lys Ser Leu Thr Pro Pro Glu Thr Ala Pro Thr Thr Asp Thr Leu
325 330 335

Ser Glu Thr Ala Val Gly Asp Ile Ser Ser Val Asp Val Asp Tyr Trp
340 345 350

Thr Arg Arg Cys Val Pro Ile Ile Gly Ser Leu Leu Lys Ser Cys Arg
355 360 365

Val Tyr Ser Glu Ala Glu Arg Glu Thr Gln Leu Arg Phe Leu Gln Glu
370 375 380

His Val Leu Pro Asn Leu Gly Pro Arg Pro Ser Ser Pro Gly Ser Gln
385 390 395 400

Ile Gln Ser Met Ala Thr Phe Ser Gly Phe Pro Leu Gln Pro Ser Ile
405 410 415

Asn Leu Ser Gly Ser Gly Gln Ala Lys Val Arg Tyr Thr Phe Glu Pro
420 425 430

Leu Asp Ser Leu Ser Gly Thr Glu Val Asp Pro Phe Ala Leu Ala Pro
435 440 445

Ala Gln Arg Val Leu Glu Lys Leu Ser Thr Leu Leu Gly Val Trp Pro
450 455 460

Gly Trp Ile Asp Ala Leu Ile Ala Ala Tyr His Pro Thr Arg Glu Glu
465 470 475 480

Val Glu Gln Leu His Pro Asn Leu His Glu Tyr Leu Arg Gly Val Leu
485 490 495

Val Arg Thr Thr Gly Arg Gln Asp Val Gln Val Pro Pro Met Pro Arg
500 505 510

Met Trp Val Cys Phe Val Ala Leu Asp Leu Glu Gly Ala Ser Gln Ala
515 520 525

Leu Lys Val Tyr Phe Asp Pro Lys Ile Lys Glu Ala Val Thr Gly Ile
530 535 540

Pro Ser Cys Lys Tyr Thr Cys Gln Ile Leu Arg Thr Val Asp Arg Phe
545 550 555 560

Gly Asn Ala Lys Ala Val Asp Met Leu Glu Gln Phe Leu Ala Glu Glu
565 570 575

His Ser Ile Gly Ala Val Glu Leu Ile Ala Ile Asp Cys Val Pro Glu
580 585 590

Glu Met Gln Pro Ser Ala Arg Ile Lys Val Tyr Val His Thr Met Ser
595 600 605

Asn Ser Phe Gln Thr Val Arg Lys Tyr Met Thr Met Gly Gly Arg Cys
610 615 620

Met Asp Pro Ala Thr Leu Glu Gly Leu Glu Asn Leu His Asp Val Trp
625 630 635 640

Tyr Ser Leu Leu Gly Glu Ser Gln Gly Ile Val Asn Glu Glu Tyr Ser
645 650 655

Lys Pro Leu Thr Gly Phe Ser Ser Met Gln His His Leu Tyr Phe Ser
660 665 670

Tyr Glu Met Thr Pro Gly Asn Ala Asp Pro Gly Val Lys Val Tyr Ile
675 680 685

Pro Val Gln Ser Tyr Ala Pro Asp Asp Lys Thr Ile Ala Gln Asn Tyr
690 695 700

Glu Ala Asn Phe Arg Gln Leu Asn Trp Pro Trp Gly Glu Pro Gly Val
705 710 715 720

Tyr Glu Ala Val Ile Glu Ser Ala Leu Gly Pro Val Lys His Ser Arg
725 730 735

Ala Thr Phe Leu His Gly Gly Ser Ser Phe Ile Phe Ser Lys Gly Arg
740 745 750

Gly Val Tyr Gln Ser Ile Tyr Leu Asp Pro Pro Leu Glu Glu Gly Gly
755 760 765

Asn Ile Ala Val Phe Glu His His Asp Asp Gln Asp Thr Ile Val Asp
770 775 780

Leu Gly Asn Met
785

<210> 54
<211> 742
<212> DNA
<213> Neotyphodium lolii

<400> 54
atggagcaag cgccgctcgc ttatcaggaa gttcaatggc tagctgaaac ttttgtcact 60

ttcatggggc ttggctggct tatcaattac gtcttgatga tctggcactc taggaggggt 120

gaaccgagca gcatggctct catacccctc tgcaacaaca tcgcctggga gctcgtatac 180

acgattatct atccgtctcc taacaaagtg gaacttgcgg ctttcatagc aggtgtcact 240

ttgaacttcc ttatcatgac ctctgcagcc cgttcggcaa gatccgagtg gagtcactca 300

cccacaatgg ctaagcatgc aggtttgatt atagtcgcag gaatattgat gtgcttcacc 360

ggacatgtag cattggcgat ggaaatagga cctgcgcttg cttactcatg gggagctgtc 420

atatgccaac tagctctaag cattggaggc gtgtgtcaat tgttgcagca gcatagtact 480

ggtgggacat catggaaact ttggtaagtg aataaatcaa ttacgtttct aatctatatt 540

gaatgtcata tcaggggtgg ctgacatgaa agttttcagg tcaagtcgat ttctaggctc 600

ttgttgtgcg gttggctttg cctttcttcg ctggagatac tggcccgagg cgtacggatg 660

gctggccagt ccccttatcc tctggagtct tgccacgttt cttgtggccg atttgacgta 720

cggggtttgt ctccttcttt ag 742

<210> 55
<211> 227
<212> PRT
<213> Neotyphodium lolii

<400> 55

Met Asp Gly Phe Ser Asn Met Glu Gln Ala Pro Leu Ala Tyr Gln Glu
1 5 10 15

Val Gln Trp Leu Ala Glu Thr Phe Val Thr Phe Met Gly Leu Gly Trp
20 25 30

Leu Ile Asn Tyr Val Leu Met Ile Trp His Ser Arg Arg Gly Glu Pro
35 40 45

Ser Ser Met Ala Leu Ile Pro Leu Cys Asn Asn Ile Ala Trp Glu Leu
50 55 60

Val Tyr Thr Ile Ile Tyr Pro Ser Pro Asn Lys Val Glu Leu Ala Ala
65 70 75 80

Phe Ile Ala Gly Val Thr Leu Asn Phe Leu Ile Met Thr Ser Ala Ala
85 90 95

Arg Ser Ala Arg Ser Glu Trp Ser His Ser Pro Thr Met Ala Lys His
100 105 110

Ala Gly Leu Ile Ile Val Ala Gly Ile Leu Met Cys Phe Thr Gly His
115 120 125

Val Ala Leu Ala Met Glu Ile Gly Pro Ala Leu Ala Tyr Ser Trp Gly
130 135 140

Ala Val Ile Cys Gln Leu Ala Leu Ser Ile Gly Gly Val Cys Gln Leu
145 150 155 160

Leu Gln Gln His Ser Thr Gly Gly Thr Ser Trp Lys Leu Trp Ser Ser
165 170 175

Arg Phe Leu Gly Ser Cys Cys Ala Val Gly Phe Ala Phe Leu Arg Trp
180 185 190

Arg Tyr Trp Pro Glu Ala Tyr Gly Trp Leu Ala Ser Pro Leu Ile Leu
195 200 205

Trp Ser Leu Ala Thr Phe Leu Val Ala Asp Leu Thr Tyr Gly Val Cys
210 215 220

Leu Leu Leu
225